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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Progesterone receptors (PR) and estrogen receptors (ER) are important prognostic indicators in breast cancer. We believe that PR, in addition to being an indicator of functional ER, can play a role in the progression of breast cancer in its own right. The two isoforms of PR, PR-A and PR-B, function differently both *in vitro* and *in vivo*; however, before our studies, few PR regulated genes had been identified and it was not known which of these progesterone-responsive genes were regulated via PR-A versus PR-B. Our studies have identified such genes and cloned some of the promoters of differentially regulated genes to use as indicators of isoform-specific PR activity. In addition, we have created breast cancer cells that inducibly express one or the other PR isoform. Using these cells, we have determined that the gene expression profile of breast cancer cell changes just by having PR even in the absence of ligand. Interestingly, we find that PR-A regulates more genes in a ligand-independent manner, whereas PR-B is the stronger ligand dependent transcription factor. We have found that PR-B uniquely regulates genes involved in mammary gland differentiation, while PR-A upregulates the anti-apoptosis gene Bcl-X_L and cells containing PR-A are more resistant to apoptosis.

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Final Report for Concept Award Number DAMD17-01-1-0507 (BC996535) to Dr. Jennifer Richer

Introduction:

Progesterone receptors (PR) in estrogen receptor (ER) positive breast tumors indicate likely responsiveness to endocrine therapies such as tamoxifen, and absence of PR is associated with hormone resistance and poor prognosis. Progesterone was added to hormone replacement therapies for post-menopausal women to counteract the proliferative effects of estrogen on the endometrium. However, although the incidence of endometrial cancer was reduced, there was a slight increase in the incidence of breast cancer. PR are expressed as two isoforms, PR-A and PR-B, with PR-B containing an extra transcriptional activation function in a 164 amino acid region in the far N-terminus that is not found in the truncated PR-A. Numerous laboratories have generated evidence that PR-B and PR-A funtion differently both *in vitro* and *in vivo* in normal breast development and in breast cancer. In order to determine if different genes are controlled by the two different forms of PR, we conducted studies with breast cancer cells containing one or the other isoform. We also made cells starting from PR negative cells that could inducibly express one or the other form. We could therefore additionally determine whether just having PR affects breast cancer cell characteristics in the presence or absence of the ligand progesterone. We hypothesized first that PR-A and PR-B regulate different genes in response to progesterone in both normal and breast cancer cells, and secondly, that as a consequence of such differential gene regulation, the presence of PR-A or PR-B will have an effect on cell differentiation, adhesion/motility, and apoptosis (cell death).

Body:

We have published two papers that resulted partially from the investigations proposed in this concept award (see references (4) and (1) attached in Appendices A and B). The first paper identifies 94 progesterone regulated genes, the majority of which are differentially regulated by the PR-A versus PR-B isoforms in response to progesterone. Some of these genes have be implicated as being important in breast cancer, while others are known to be important in mammary gland development. For instance we find that PR-B regulates genes known to be responsible for mammary gland differentiation such as Stat5a and C/EBPβ (see Figure 4 in reference (4), Appendix B). On the other had some genes that we find to be uniquely regulated by PR-A could be deleterious in breast cancers, such as Bcl-X_L and HEF1 (see figure 5 in (4). The anti-apoptosis gene, Bcl-X_L is upregulated only by PR-A. Resistance to apoptosis by preferential upregulation of Bcl-X_L could explain the deleterious effect of PR-A overexpression in the mammary gland of transgenic mice (6). HEF1, a docking protein associated with focal adhesion kinase (9), is also preferentially upregulated by PR-A. HEF1 is related to BCAR1/p130Cas, which is upregulated in tamoxifen resistant tumors (7, 8). This led us to ask whether tumors expressing only PR-A might be more resistant to apoptosis-inducing chemotherapeutic agents such as taxol or to tamoxifen? Taken together, our data raise the possibility that physiological progesterone levels are harmful in breast cancer, and may explain recent hormone replacement therapy (HRT) studies indicating that unlike its effect in the uterus, progesterone is not protective in the breast, and indeed increases breast cancer risk (3, 5). Thus, in this first publication, we identified genes known to be important in

differentiation and resistance to apoptosis which are regulated differentially by PR-B versus PR-A. This first large-scale study of PR gene regulation has important implications for the measurement of PR in breast cancers and for the many clinical uses of synthetic progestins: first, that it is important to distinguish between the two isoforms in breast cancers and second, that isoform-specific genes can be used to screen for ligands that selectively modulate the activity of PR-A versus PR-B. In the past, consensus response elements rather than relevant target genes, have generally been used to study nuclear hormone receptor activity, possibly skewing our understanding of hormone receptor action.

The second publication (1) (attached as Appendix B) describes the construction of breast cancer cells that inducibly express one or the other isoform of PR. Additionally cells were made that constitutively express one isoform with the other isoform being inducible, allowing us to investigate effects of altering the PR-A to PR-B ratio. Constructing these inducible cells was one of the tasks that we set out to accomplish in the Concept Award. These cells allow us to very tightly induce the progesterone receptor in a dose dependent manner under the control of the inducer ponasterone A (see figures 1 and 2 in reference (1) Appendix B. We found that just having PR (either PR-A or PR-B) changed the gene expression profile even in the absence of progesterone. This is an important finding because PR is known to be an important marker of a functional estrogen receptor (ER) in breast cancer, but tumors that have both ER and PR are known to respond better to tamoxifen than those that have ER alone. Many of these tumors exist in postmenopausal women that have little circulating progesterone. PR was not traditionally thought to act in a ligand independent manner. However, we have proven, using these cells in which PR can be turned off or on in the same cells, that the mere presence of PR causes alteration in gene expression. Interestingly, we observe that although PR-B is the stronger transcriptional activator in the presence of ligand (which makes since as it has an extra activation function), it is PR-A that regulates the majority of genes in a ligand –independent manner.

We also had as a goal in the concept award grant to clone the promoters of some of the genes that are differentially regulated by the two progesterone receptor isoforms. We have accomplished this goal and now have the integrin α 6 promoter, which is specifically activated by PR-B, as well as the PR-A specific Bcl-X promoter, linked to a luciferase reporter. Data from these promoters is shown in figures 3 and 5 in (4). These genes, as well as other isoform-spedific genes that we have identified, can be used to screen synthetic progestins that selectively activate one or the other form of the progesterone receptor. This is important because work with PR-A and PR-B knockout mice has shown that the PR-A form is probably the form that is responsible for inhibiting the proliferative effect of estrogen in the endometrium, while it is the PR-B isoform that is important for both proliferation and differentiation in the mammary gland (2).

Since the anti-apoptosis gene Bcl-X_L is upregulated by PR-A, we examined the role of progesterone in apoptosis in PR-A versus PR-B containing cells. Using a cell death detection ELISA assay (Roche Molecular Biochemicals) which measures the extent of apoptosis based on the amount of DNA degredation, we observed that PR-A expressing cells are more resistant to apoptosis in general. In the presence of progesterone, significantly less apoptosis occurs in both cell lines (Figure 1 below). In otherwords, progesterone protects against apoptosis. This fits with progesterone inducing

anti-apoptosis genes and particularly with our finding in figure 5 in reference (4), Appendix A, that PR-A containing cells having more abundant basal levels of Bcl-X_L, and progesterone inducing more Bcl-X_L in PR-A containing cells as compared to PR-B containing cells. Additionally, we observed that in the presence of progesterone, PR-A expressing cells are more resistant to the chemotherapeutic agent taxol than cells that contain PR-B. In addition, progesterone inhibited apoptosis induced by the chemotherapeutic drug taxol (although this trend was not statistically significant due to variation among replicates, it was repeatable) (figure 1 below). We used taxol to induce apoptosis instead of tumor necrosis factor (TNF) (as initially proposed in the grant) because it turns out that these particular breast cancer cells are relatively resistant to TNF. These studies are still underway with the inducible cells. We will also try to block this antiapoptotic phenotype with antisense RNAs to Bcl-X_L or some of the other antiapoptosis proteins that we found to be induced by progesterone.

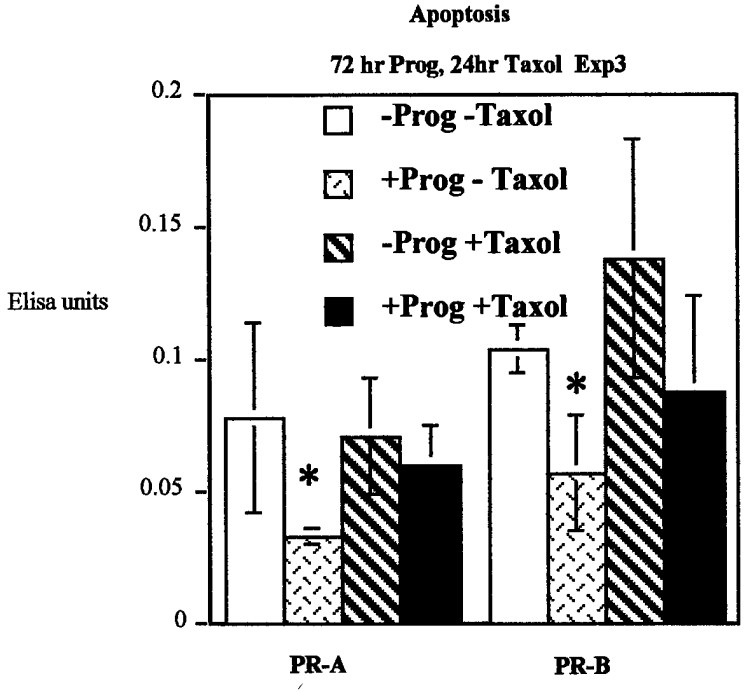

Figure 1. Apoptosis in breast cancer cells expressing PR-A versus PR-B. *Cells containing PR-A or PR-B isoforms were treated with either no progesterone, no taxol (ethanol vehicle only) in open bars; progesterone, no taxol, red; no progesterone, plus taxol, green stripes; and progesterone and taxol, blue bars. Total time of progesterone treatment was 72 hours, and taxol was added for the last 24 hours. * Denotes statistical difference progesterone treated and untreated (no progesterone or taxol) groups by ANOVA at $p<0.05$*

We also are in the process of examining migration of PR-A compared to PR-B containing T47D breast cancer cells using a Boyden chamber assay as proposed in the grant. These studies are just beginning as we have just now finished fully characterizing the inducible cells and getting the Boyden chamber assay set up. A year was not quite enough time to make the cell lines and get these assays set up and have reportable data.

Although we do see important genes and their protein products that are known to be involved in mammary gland lobuloalveolar differentiation upregulated by PR-B specifically, we have not yet looked at the effect of PR-A versus PR-B isoform content on differentiation of cells on extracellular matrix (ECM) because we wanted to do this with normal breast epithelial cells, the MCF-12A cells. These cells will actually organize into ducts when cultured on ECM. In the original grant we proposed to stably reintroduce the PR isoforms individually into these cells. This was attempted, however we could not get any clones that expressed PR at high enough levels to use. In culture normal cells

loose their estrogen and progesterone receptors. In the future we may have to use adenovirus to get the normal cells to express PR.

Key Research Accomplishments:

1) Completed microarray studies identifying genes differentially regulated by PR-B versus PR-A. Reference (4) and Appendix A
2) Completed engineering of breast cancer cells that inducibly express one or the other PR isoform. Reference (1) and Appendix B
3) Completed additional microarray experiments with inducible PR isoform cells confirming genes differentially regulated in the presence of ligand and also making the novel observation that some genes change just by virtue of having PR even in the absence of ligand. Reference (1), Appendix B
3) Completed and/or initiated preliminary studies on:
    a) differentiation (described in (4))
    b) apoptosis (see figure 1 of this report)
    c) migration (not finished)

Reportable Outcomes:

1) Publication of two papers:
    (4) Appendix A
    (1) Appendix B
2) Presentation at 4 national meetings, abstracts in Appendix C
    Keystone Nuclear Hormone Receptor Superfamily 2002 Abstract # 253 , Mammary Gland Gordon Conference 2002 (abstracts not published), and The Endocrine Society, oral presentation abstract # OR41-3.
3) Production of 4 cell lines derived from human T47D breast cancer cells inducibly expressing either PR-A, PR-B, or constitutively expressing PR-A with inducible PR-B or vice-versa.
4) Cloning of isoform-specific gene promoters linked to luciferase reporters to use as tools for screening isoform specific ligands.
5) Clinical Bridge Award from the DOD Breast Cancer Research Program to Dr. Jennifer Richer based on experience/preliminary data supported by this award.

Conclusions:

This first large-scale study of PR gene regulation has important implications for the measurement of PR in breast cancers and for the many clinical uses of synthetic progestins. First we have learned that it is important to distinguish between the two isoforms of PR in breast cancers because they behave very differently and currently used clinical assays do not distinguish between the two. Secondly we have identified isoform-specific genes that can be used to screen for ligands that selectively modulate the activity of PR-A versus PR-B. In the past, synthetic consensus response elements, rather than real and relevant target genes, have generally been used to study nuclear hormone receptor activity, possibly skewing our understanding of their action.

Synthetic progestins are used in hormone replacement therapy which has been reported to slightly increase the risk of breast cancer when progestins were added. The PR-A isoform has been found to be responsible for inhibiting the proliferative effect of

estrogen in the endometrium, while it is the PR-B isoform that is important for both proliferation and differentiation in the mammary gland. Therefore, a PR-A specific ligand added to HRT regimen might be effective in blocking the proliferative effects of unopposed estrogen in the endometrium (reducing the risk of endometrial cancer), while not driving proliferation in the breast and increasing the incidence of breast cancer.

In our second paper in which we made cells in that inducibly express either PR-A or PR-B, we concluded that the presence of the receptors could change the gene expression profile even in the absence of progesterone. This is an important finding because PR is known to be an important marker of a functional estrogen receptor (ER) in breast cancer, but tumors that have both ER and PR are known to respond better to tamoxifen than those that have ER alone. Many of these tumors exist in postmenopausal women that have little circulating progesterone. PR was not previously thought to act in a ligand independent manner.

Finally the research supported by this concept award DAMD17-01-1-0507 has provided preliminary data that was included in a Clinical Bridge Award to investigate, amongst other things, whether PR isoform content differs in tumors that respond to endocrine therapies versus those that do not.

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Appendix A

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Differential Gene Regulation by the Two Progesterone Receptor Isoforms in Human Breast Cancer Cells*

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The PR-A and PR-B isoforms of progesterone receptors (PR) have different physiological functions, and their ratio varies widely in breast cancers. To determine whether the two PR regulate different genes, we used human breast cancer cell lines engineered to express one or the other isoform. Cells were treated with progesterone in triplicate, time-separated experiments, allowing statistical analyses of microarray gene expression data. Of 94 progesterone-regulated genes, 65 are uniquely regulated by PR-B, 4 uniquely by PR-A, and only 25 by both. Almost half the genes encode proteins that are membrane-bound or involved in membrane-initiated signaling. We also find an important set of progesterone-regulated genes involved in mammary gland development and/or implicated in breast cancer. This first, large scale study of PR gene regulation has important implications for the measurement of PR in breast cancers and for the many clinical uses of synthetic progestins. It suggests that it is important to distinguish between the two isoforms in breast cancers and that isoform-specific genes can be used to screen for ligands that selectively modulate the activity of PR-A or PR-B. Additionally, use of natural target genes, rather than "consensus" response elements, for transcription studies should improve our understanding of steroid hormone action.

Progesterone receptors (PR)[1] are ligand-activated transcription factor members of the steroid hormone family of nuclear receptors. They exist naturally as two isoforms, PR-B and PR-A, transcribed from two promoters on a single gene (1). Human PR-B are 933 amino acids in length and contain a unique activation function AF3 (2). PR-A lack the 164 N-terminal residues that contain AF3 and are 769 amino acids in length. Both isoforms are physiologically important. Mice lacking both PR display pleiotropic reproductive abnormalities, incomplete mammary gland development, and impaired thymic function and sexual behavior (3), whereas those lacking only PR-A exhibit a subset of these phenotypes (4).

Clinically, PR are important therapeutic targets. Progestational agents are widely used for oral contraception, menopausal hormone replacement therapy (HRT), and to treat breast cancer and endometrial hyperplasia (5, 6). Antiprogestins are in clinical trials for contraception, induction of labor, and the treatment of meningiomas, endometriosis, and endometrial cancers. In breast cancers, total PR levels are routinely measured as a guide to hormone therapy and as markers of disease prognosis (7–10). Interestingly, whereas progestins added to HRT successfully decrease the incidence of endometrial cancer, they increase the incidence of breast cancer (11, 12).

Little is known regarding the unique roles of the two PR isoforms in progesterone target tissues. *In vitro*, the two receptors have markedly different transcriptional effects on progestin-responsive promoters (2, 13–16). The antiprogestin RU486 has partial agonist effects only on PR-B, whereas only PR-A inhibit PR-B and other steroid receptors including estrogen receptors (ER) (17–19). *In vivo*, the two PR isoforms are usually coexpressed in normal cells, yet their ratio varies dramatically in different tissues, physiological states, and in disease (20–22). For example, in the estrogen-treated primate, the hypothalamus expresses an excess of PR-B, but the pituitary expresses an excess of PR-A (23, 24). In human endometrium the levels and ratio of PR-A to PR-B vary extensively during the menstrual cycle (25–28), and overexpression of PR-B is associated with highly malignant forms of endometrial, cervical, and ovarian cancers (29, 30).

With regard to the mammary gland, in transgenic mice, 3:1 overexpression of PR-A over PR-B results in extensive epithelial cell hyperplasia, excessive ductal branching, and a disorganized basement membrane, all features associated with neoplasia (31). In contrast, overexpression of PR-B leads to premature ductal growth arrest and inadequate lobulo-alveolar differentiation (32). Interestingly, PR-A null mice, which express only PR-B, exhibit normal mammary gland development, yet the same mice display severe uterine hyperplasia and reproductive defects (4). Collectively, these data suggest that PR-A and PR-B have physiologically different functions in different tissues and that alterations in their ratios carry different consequences depending on the tissue.

Although PR levels are routinely measured in breast cancers for clinical decision making, only two studies have examined the levels of the two isoforms. An analysis of 202 PR-positive

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[1] The abbreviations used are: PR, progesterone receptor(s); HRT, hormone replacement therapy; ER, estrogen receptor(s); ERR, estrogen-related receptor; MEM, minimum essential medium; RT, reverse transcriptase; PM, perfectly matched; MM, mismatched; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; β2MG, β_2-microglobulin; MCP, monocyte chemotactic protein; βHSD, 11β-hydroxysteroid dehydrogenase; AF, activation function.

breast cancers by immunoblotting shows that expression levels of PR-A are higher than PR-B in 59% of tumors and are 4-fold or greater in 25% of tumors (33). In another study of 32 PR-positive breast cancers, excess PR-B correlated with the absence of Her-2/neu indicating a good prognosis, whereas excess PR-A correlated with a poorly differentiated phenotype and higher tumor grade (34). Overexpression of PR-A in cultured human breast cancer cells results in marked morphological changes and loss of adherent properties (35), suggesting, as do the transgenic mice data, that an excess of PR-A is particularly harmful in the breast.

Little is known at present about the molecular mechanisms that might explain these differences. We therefore undertook the first systematic, large scale comparison of gene regulation by the two PR, using a unique human breast cancer cell model for this purpose. Wild-type T47Dco breast cancer cells express equimolar levels of PR-A and PR-B in an estrogen-independent manner (36). To study differential gene regulation by the two PR isoforms independently, we isolated a PR-negative subline of T47Dco (designated T47D-Y cells) and then engineered the T47D-Y to stably express equivalent levels of either PR-B (T47D-YB cells) or PR-A (T47D-YA cells) (37). Because these are pure cell populations, and all of the cells have the same parental-cell background, the PR isoform-specific effects of progesterone on gene transcription can be quantitatively and reproducibly investigated in a tightly controlled manner.

Our data, based on triplicate determinations, demonstrate that in response to progesterone, PR-A and PR-B primarily regulate different subsets of genes, and although PR-B are transcriptionally more active, there are genes that are uniquely regulated by PR-A. These subsets include genes known to be involved in breast cancer and/or mammary gland development but not previously known to be progesterone targets. Progesterone regulation of many of these genes would be deleterious in breast cancers. A surprisingly large number of genes are targeted to the cell membrane or involved in membrane-initiated signaling. Other gene clusters are involved in metabolism, transcription, cell growth and apoptosis, and nucleic acid and protein processing. The results suggest that PR-A and PR-B have different molecular functions and that it may be important to quantify either the PR isoform content of breast cancers or their gene targets, rather than total PR.

EXPERIMENTAL PROCEDURES

Cell Culture—The PR-positive T47Dco breast cancer cell line, isolation of its PR-negative clonal derivative T47D-Y, and construction of T47D-YA and T47D-YB cells have been described (36, 37). Cells are routinely cultured as described previously (38).

Atlas™ Human cDNA Expression Array—T47D-YA and T47D-YB cells were grown to ~70% confluence in Eagle's minimum essential medium (MEM) with Earle's salts as described previously except without G418, washed with serum-free MEM, and changed into MEM containing 5% charcoal-stripped fetal calf serum for 24 h, after which the cells were treated with 10 nM progesterone dissolved in ethanol or with ethanol alone, for 6 or 12 h. Total RNA was prepared from the 4 sets of cells using guanidinium isothiocyanate; poly(A)$^+$ RNA was purified with the Oligotex mRNA kit (Qiagen, Valencia, CA), and ^{32}P-labeled cDNA was synthesized from 1 μg of each sample using SuperScript II reverse transcriptase (Invitrogen). Labeled probes were hybridized to Atlas™ Human cDNA Expression Array (CLONTECH, Palo Alto, CA) nylon membranes onto which 588 cDNA fragments encoding known proteins are spotted in duplicate. After processing according to the CLONTECH User Manual (PT3140-1 PR91208), signals were detected by PhosphorImager™ (Molecular Dynamics, Sunnyvale, CA). Data were analyzed using Atlas™ Image 1.0 (CLONTECH) and normalized to signals from control housekeeping genes on the same filter. For selected genes, progesterone inducibility and PR isoform specificity were confirmed by reverse transcriptase (RT)-PCR, and/or Western blotting as described below.

Affymetrix GeneChip™ Experiments—T47D-Y, T47D-YA, and T47D-YB cells were grown to ~70% confluence in MEM without antibiotic, washed with serum-free media, and changed into media containing 5% charcoal-stripped fetal calf serum for 24 h. Cells were then treated with 10 nM progesterone dissolved in ethanol, or in ethanol alone, for 6 h. Total RNA and poly(A)$^+$ RNA were prepared from the 6 samples as described above. Poly(A)$^+$ RNA was processed according to the Affymetrix Expression Analysis Technical Manual (P/N 700218 rev2). Briefly, first strand and second strand cDNA syntheses were performed, and biotin-labeled cRNA was generated using the EnZo BioArray™ High Yield Transcript Labeling Kit (Enzo Diagnostics, Inc., Farmingdale, NY). Unincorporated nucleotides were removed with RNeasy affinity columns (Qiagen, Valencia, CA). Purified, biotinylated cRNAs were quantified, and 20 μg were subjected to a fragmentation reaction to randomly generate fragments ranging from 35 to 200 bases. HuGeneFL 6800 Array™ chips consisting of 5,600 full-length human genes from Unigene, GenBank™, and TIGR data bases were used for hybridization. Thirty μl of fragmented cRNA were added to a hybridization mixture together with control oligonucleotide B2 and control cRNA mixture, then washed, and stained with streptavidin/phycoerythrin. DNA chips were read at a resolution of 6 μm with a Hewlett-Packard GeneArray Scanner. The entire experiment was performed three separate times with PR-positive T47D-YA and T47D-YB cells and two separate times with PR-negative T47D-Y cells. Each repeat was separated by ~1 month and was designed to be a true replicate taking into account experimental variability in cell culture conditions and sample preparation. To determine which progesterone-regulated genes are direct targets of PR, a separate experiment was performed in which T47D-YB cells were treated with cycloheximide (10 μg/ml) 30 min before treatment with or without 10 nM progesterone. Cells were otherwise treated as described above, and RNA was derivatized and hybridized to microarray chips as above.

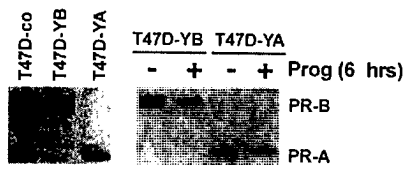

FIG. 1. **Expression of PR-A and PR-B isoforms in T47Dco, T47D-YB, and T47D-YA cells.** 100 μg of whole cell lysate from parental T47Dco cells (expressing PR-A and PR-B), T47D-YB cells (expressing PR-B only), and T47D-YA cells (expressing PR-A only) were resolved by SDS-PAGE and immunoblotted with AB-52 antibody, which recognizes both isoforms of PR (*left panel*). 100 μg of whole cell lysate from T47D-YB and T47D-YA, cells treated without (−) or with (+) 10 nM progesterone (*Prog*) were resolved by SDS-PAGE and immunoblotted with AB-52 (*right panel*).

Data Analyses and Statistics—Detailed protocols for data analyses of Affymetrix microarrays and extensive documentation of the sensitivity and quantitative aspects of the method have been described (39). Briefly, MicroArray Suite 4.0 Expression Analysis Program™ (Affymetrix, Inc., Santa Clara, CA) was used for the first level of analysis, including the "present" or "absent" call, and pairwise comparisons. Each gene on the chip is represented by perfectly matched (PM) and mismatched (MM) oligonucleotides from 16 to 20 regions of the gene. The number of instances in which the PM hybridization signal is larger than the MM signal is computed along with the average of the logarithm of the PM:MM ratio (after background subtraction) for each probe set. These values were used to arrive at a matrix-based decision concerning the presence or absence of an RNA transcript. The average difference serves as a relative indicator of the level of expression of a transcript and is used to determine the change in the hybridization intensity of a given probe set among different experiments. Multiple experimental (minus *versus* plus progesterone) pairwise comparisons were performed. In addition, multiple control comparisons (all minus hormone samples and all plus hormone samples) were performed to serve as a measure of the variability among samples. Finally, we compared fold change in "minus" versus "plus" hormone sets in PR-positive cells to fold change in PR-negative controls.

The data were also analyzed using GeneSpring™ version 4.0 (Silicon Genetics, San Carlos, CA) to identify patterns of gene regulation in PR-A, PR-B, or PR-negative cells treated with or without progesterone. To normalize for staining intensity variations among chips, the average difference values for all genes on a given chip were divided by the median of all measurements on that chip. In addition, to scale the gene expression measurements so that they could be plotted on a reasonable *y* axis for visualization in GeneSpring™ 4.0, the average difference value for each individual gene was then normalized to itself by dividing

TABLE I

Genes regulated by progesterone organized by primary function of gene product

Tilde indicates genes absent in one or the other sample (absent without hormone in the case of up-regulated genes or absent with hormone in the case of down-regulated genes).

Accession no.	Gene name	Regulation pattern	-Fold
Membrane effects			PR-B,PR-A
Cell adhesion or cytoskeletal interactions			
X51521	*VIL2*[a]	Up by both	12.7, 5.4
HG2743-HT2846[b]	*CALD*	Up by PR-B	~5.1
L43821[b]	*HEF1*	Up by PR-A	4.7
U40572[b]	*SNTB2*	Up by PR-B	4.6
X53586	*ITGA6*[a]	Up by PR-B	2.5
X98534[b]	*VASP*	Up by both	2.5, 2.0
U32944	*PIN*	Up by PR-B	2.1
U90878	*PDLIM1*	Up by PR-B	2
X79683[b]	*LAMB2*	Down by PR-B	−2.6
Calcium-binding proteins			
D16227[b]	*HPCAL1*	Up by both	~4, 3.5
X65614	*S100P*	Up by both	3.6, 2.4
Secreted molecules			
L13720[b]	*GAS6*	Up by PR-B	~23.1
M27436[b]	*F3*	Up by PR-B	~18.1
M68516[b]	*SERPINA5*	Up by PR-B	~6.2
M77140[b]	*GAL*	Up by PR-B	~6
M23254[b]	*CAPN2*	Up by PR-B	3.6
Cytokines/cytokine receptors and chemokines			
M27492	*IL1R1*	Down by PR-B	~−3.4
J03241	*TGFB3*	Down by both	~−1.8, ~−3
HG4069-HT4339[b]	*SCYA8*	Down by both	~−7.3, ~−7.4
Membrane-bound molecules			
U16799[b]	*ATP1B1*	Up by both	6.1, 3.9
U83461[b]	*SLC31A2*	Up by PR-B	3.7
U33632[b]	*KCNK1*	Up by PR-B	2.6
M84349[b]	*CD59*	Up by PR-B	1.7
M69225	*BPAG1*	Down by both	~−3.5, ~−2.4
J65011	*PRAME*	Down by both	~−2.5, ~2.0
Signal transduction from membrane			
D79990	*RASSF2*	Up by PR-B	10.2
M69043	*NFKBIA*	Up by both	4.2, 2.0
U02081	*NET1*[c]	Up by PR-B	4.1
HG2167-HT2237[b]	*AKAP13*	Up by PR-B	~3.5
X60673[b]	*AK3*	Up by both	3.4, 1.9
HG2530-HT2626	*CAP2*	Up by both	2.9, 1.8
D86962[b]	*GRB10*	Up by both	2.9, 2.5
D10704	*CHK*	Up by PR-B	2.6
U14603[b]	*PTP4A2*	Up by both	2.3, 1.8
M18391	*EPHA1*	Down by PR-B	~−2.3
U44103	*RAB9*	Down by both	−2.8, −2.8
Metabolism			
Cholesterol or steroid metabolism and trafficking			
U26726[b]	*HSD11B2*	Up by both	~22.6, ~6.5
D17793	*AKR1C3*	Up by PR-B	~4
AF002020	*NPC1*	Up by PR-B	2.1
Fatty acid/lipid metabolism			
M76180[b]	*DDC*	Up by PR-B	~6.5
D50840	*UGCG*	Up by both	~5.6, ~2.9
S69189	*ACOX1*	Up by PR-B	~4.5
X52730[b]	*PNMT*	Up by PR-B	4.4
L09229	*FACL1*	Up by PR-B	3.3
U09646[b]	*CPT2*	Up by PR-B	3.2
X59834[b]	*GLUL*	Up by PR-B	3.1
D78335[b]	*UMPK*	Up by PR-B	3.1
Y08134	*ASML3B*	Up by PR-B	2.6
J02888	*NMOR2*	Up by PR-B	2.3
Y08682	*CPT1B*	Down by PR-B	−3.1
Nucleotide or amino acid metabolism			
M37400[b]	*GOT1*	Up by both	3.1, 1.8
U97105[b]	*DPYSL2*	Up by PR-B	2
U07225[b]	*P2RY2*	Down by PR-B	~−4.3
General metabolic/synthetic			
U01120[b]	*G6PC*	Up by PR-B	~9.8
U07919	*ALDH1A3*	Up by PR-B	3
M21154	*AMD1*	Up by PR-B	2.5
M95767	*DTBS*	Up by PR-B	~2.5
J05459	*GSTM3*	Up by PR-B	1.9
D25328	*PFKP*	Up by both	1.9, 1.9

TABLE I—*continued*

Accession no.	Gene name	Regulation pattern	-Fold
Nucleic acid and protein processing			
DNA replication/transcription/translation and protein processing			
U76421	*ADARB1*	Up by PR-B	~4.7
U41387	*DDX21*	Up by PR-B	3
L18960[b]	*EIF1A*	Up by PR-B	2.4
L41887	*SFRS7*	Up by PR-B	2.3
U77949[b]	*CDC6*	Up by PR-B	2.5
Chaperones/protein folding			
U42031[b]	*FKBP54*	Up by both	9.4, 3.3
M80254	*PPIF*	Up by PR-B	1.9
Cell cycling and apoptosis			
U37546[b]	*BIRC3*	Up by PR-B	~7.2
U45878[b]	*BIRC2*	Up by PR-B	~3.3
Z23115	*Bcl-X$_L$*[c]	Up by PR-A	3.2
D87953[b]	*NDRG1*	Up by PR-B	6.8
S81914	*IEX-1*	Up by both	2.6, 2.1
M15796	*PCNA*	Up by PR-B	1.6
X61123[b]	*BTG1*	Up by PR-B	2.2
M92287[b]	*CCND3*	Up by PR-B	2.2
Transcription factors			
U70663[b]	*EZF*	Up by both	~6.0, ~7.5
M83667[b]	*NF-IL6 (C/EBPβ)*	Up by PR-B	6.4
U43185[b]	*STAT5A*	Up by PR-B	~6.1
U44754	*SNAPC1*	Up by PR-B	4.4
D15050	*TCF8 (AREB6)*	Up by PR-B	3.6
D31716[b]	*BTFB*	Up by PR-B	3.2
U00115[b]	*BCL6*	Up by both	2.4, 2.3
M95929	*PMX1*	Up by both	2.1, 2.0
D89377[b]	*MSX2*	Up by PR-B	2
Z50781[b]	*DSIPI*	Up by PR-A	2.5
L38487	*ESRRA (ERRα1)*	Up by PR-A	2.3
Unknown function			
D25539[b]	*KIA0040*	Up by PR-B	~8
D31887[b]	*KIAA0062 related to LIV1*	Up by PR-B	3.5
U79288[b]	*Clone 23682*	Up by PR-B	2.8
D83781	*KIAA0197*	Up by PR-B	2.5
D80001	*KIAA0179*	Up by PR-B	2.4
D63487	*KIAA0153*	Up by PR-B	2.3
D79994[b]	*KIAA0172 (similar to ankyrin)*	Up by PR-B	2.1
M92357[b]	*TNFAIP2*	Down by PR-B	−2.1
U90907	*Clone 23907*	Down by both	−2.1, −2.1

[a] Denotes genes detected using Clontech™ Atlas arrays.

[b] Indicates genes regulated by progesterone in the presence of cycloheximide.

[c] Denotes detected by both Affymetrix™ and Clontech™ arrays.

all measurements for that gene by the mean of the expression values of the gene over all the samples. Normalized values below 0 were set to 0. Finally, to identify patterns of gene expression among cell lines and hormone treatments, *k*-means clustering was performed generating 24 clusters representing 53.8% explained variability. This generated clustergrams of genes regulated by progesterone in a PR isoform-dependent manner. However, because replicates were done for each cell line, additional statistical analyses were possible, and genes whose regulation was not statistically significant were discarded from the clusters. Statistical significance was assessed by one-way analysis of variance using a cut-off value of $p < 0.05$, followed by a Tukey multiple comparison test to determine whether the expression level in any individual cell line or hormone treatment was different from all other expression levels. The genes shown in the figures and listed in the tables were statistically significantly regulated by progesterone or, in the case of the ligand-independent effects, were significantly different in the presence *versus* the absence of PR.

RT-PCR—PCR amplifications included coamplification of internal controls, either β_2-microglobulin (*β2MG*) or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). Control primers are as follows (where fwd is forward and rev is reverse): *β2MG* fwd, 5′-atccagcgtactccaaagattc-3′, and *β2MG* rev, 5′-tccttgctgaaagacaagtctg-3′ (178 bp); *GAPDH* fwd (447 bp), 5′-ccatgttcgtcatgggtgtgaacca-3′, or *GAPDH* fwd (633 bp), 5′-ggctctccagaacatcatccctgc-3′, and *GAPDH* rev (932 bp), 5′-gggtgtcgctgttgaagtcagagg-3′, to yield products of 485 or 299 bp. Other primers are as follows: *HEF1* fwd, 5′-actgtcagcctccccagctcaggacaa-3′, *HEF1* rev, 5′-atcgtcacacttgttctggggctt-3′; *ERR* fwd, 5′-ctgggtgtggcccagcgctcactg-3′, *ERR* rev, 5′gccagcccggccggcttcatactc-3′; *MCP* fwd, 5′-gatctcagtgcagag-

TABLE II
Genes encoding products previously reported to be regulated by progesterone

Accession no.	Gene name	Cell or tissue type
U26726	*HSD11B2*	Breast cancer cells, endometrial cancer cells
M27436	*F3*	Endometrium
U42031	*FKBP54*	Breast cancer cells
M68516	*SERPINA5*	Endometrial stromal cells
U43185	*STAT5A*	Breast cancer cells
U16799	*ATP1B1*	Breast cancer cells
X52730	*PNMT*	Adrenal medulla
M69043	*NFKBIA*	Macrophage cells and endometrium
U35048	*TSC22*	Breast cancer cells
AF002020	*NPC1*	Granulosa cells
D25328	*PFKP*	Breast cancer cells, intestine, granulosa cells
M80254	*PPIF*	Liver
X53586	*ITGA6*	Breast cancer cells
Z23115	*Bcl-X_L*	Endometrial cells
HG4069-HT4339	*SCYA8*	Endometrial cells and breast cancer cells

gctcg-3′, MCP *rev*, 5′-tgcttggtccaggtggtccat-3′; *HSD11β2* fwd, 5′-catcgagcacttgcatgggcagtt-3′, *HSD11β2* rev, 5′-ccaggctggccaggctgcagtgct-3′; *BPAG* fwd, 5′-gatgacaggaatttctagcctctac, *BPAG* rev, 5′-cacgcagttgaaggctgtgggaacg-3′; *TF* fwd, 5′-cacagagtgtgacctcaccgacgag-3′, *TF* rev, 5′-gtactcttccggttaactgttcgg-3′; integrin α_6 fwd, 5′-cctgaggactgatttcagagtgactaca-3′, integrin α_6 rev, 5′-tcttgtgatgtgggacagctaacgtgat-3′; *BCL-X_L* fwd, 5′-caggcgacgagtttgaactgcggtac-3′, BCL-X_L rev, 5′aaggctctaggtggtcattcaggtaagt-3′; S100P fwd, 5′-gtgctgatggagaaggagctacca-3′, S100P rev 5′-taatcagaggtacatgagcaggct-3′; *EZF* fwd, 5′-ggcccaattacccatccttcctgc-3′, *EZF* rev, 5′-tgtgtaaggcgaggtggtccgacctgg-3′. All primers were designed to anneal at a temperature of 65 °C for specificity and to produce products between 200 and 600 nucleotides. Total RNA was prepared from cells treated with progesterone or ethanol vehicle for 3–48 h. One μg of total RNA was mixed with 0.4 μM random hexamers and heated to 65 °C for 5 min. 1× PCR buffer (5 mM $MgCl_2$), 20 units of RNase inhibitor, 4 mM dNTPs, and 125 units of Moloney murine leukemia virus reverse transcriptase were added, and tubes were incubated at 42 °C for 1 h. Five μl of the cDNA synthesis reactions were added to 1× PCR buffer, 1.8 mM $MgCl_2$, 10 mM dNTP blend, and 60 pmol of specific primers were incubated with 5 units of AmpliTaq DNA polymerase at 94 °C for 30 s, 65 °C for 45 s, and 68 °C for 1 min for 16–18 cycles. Cycle numbers were determined to be in the linear range of amplification for each product by removal of 4 μl of product every 3 cycles, followed by densitometric quantification of each product over a 8–30 cycle range. (PCR reagents were from PerkinElmer Life Sciences.) Five μl of products were resolved on a 2% agarose gel, and Southern blots were performed in 0.4 M NaOH. Blots were prehybridized in Rapid-Hyb (Amersham Biosciences) for 1 h at 65 °C. cDNA probes were generated by RT-PCR and radioactively labeled with [α-^{32}P]dCTP using MegaPrime DNA labeling system (Amersham Biosciences). Blots were probed for 2 h to overnight at 65 °C, washed, and exposed to autoradiography film or PhosphorImager screen. In some cases, RT-PCR products were visualized and quantitated directly on an ethidium bromide-stained gel.

Transcription Assay—HeLa cells plated at 1 × 10^5 cells per 60-mm dish in MEM supplemented with 5% fetal calf serum were transiently transfected by $CaPO_4$ coprecipitation with 100 ng of hPR1 (PR-B expression vector) or hPR2 (PR-A expression vector; gifts of Pierre Chambon, Strasbourg, France) and either 1.2 μg of the integrin α_6 promoter (to −760 nucleotides) cloned upstream of a luciferase reporter (gift of Sohei Kitazawa, Kobe University School of Medicine, Japan) or 1.2 μg of pA3(−1011/−1)BCL-X_L-LUC construct (cloned as described below), 1.2 μg of β-galactosidase expression plasmid pCH110, and 5.5 μg of Bluescribe carrier plasmid. Cells were treated with 10 nM progesterone or ethanol vehicle. After 24 h of treatment, cells were harvested in lysis solution, and 60 μl of lysate were analyzed for luciferase activity using the Enhanced Luciferase Assay Kit (Analytical Luminescence Laboratories, Ann Arbor, MI) and a Monolight 2010 Luminometer, and relative luciferase units were corrected for transfection efficiency using the β-galactosidase control. To clone the pA3(−1011/−1)*BCL-X_L-LUC*, a human genomic library (CLONTECH catalog number HL1067J) was screened with a [α-^{32}P]dCTP-labeled 246-bp *BCL-X_L* 5′ PCR fragment. DNA was isolated from positive phage clones, and the *BCL-X_L* promoter fragment was amplified by PCR using T7 and *BCL-X_L* (5′-ttttataatagggatgggctcaa-3′) primers. The blunt-ended PCR product was subcloned into the pA3LUC vector (gift of William Wood, University of Colorado Health Science Center, Denver, CO) and sequenced.

TABLE III
Genes encoding products involved in breast cancer or mammary gland development

Accession no.	Gene name
L13720	*GAS6*
M27436	*F3*
M83667	*NF-IL6 (C/EBP$_\beta$)*
M68516	*SERPINA5*
U43185	*STAT5A*
X65614	*S100P*
X53586	*ITGA6*
D89377	*MSX2*
M69225	*BPAG*
U70663	*EZF*

Immunoblots—Cells were plated at 1 million per 100-mm^2 plates, treated with 10 nM progesterone for the times indicated, and harvested in RIPA buffer as described previously (38). Protein extracts were equalized to 150 μg by Bradford assay (Bio-Rad), resolved by SDS-PAGE, and transferred to nitrocellulose. Equivalent protein loading was confirmed by Ponceau S staining. Following incubation with the appropriate antibodies and horseradish peroxidase-conjugated secondary antibodies, protein bands were detected by enhanced chemiluminescence (Amersham Biosciences). Primary antibodies were STAT5 C-17 (detects both STAT5A and 5B isoforms), p21 (C-18), C/EBPβ (Δ 198) (specific for LAP isoforms), and C/EBPβ (C-19) (detects both LAP and LIP isoforms), all from Santa Cruz Biotechnology (Santa Cruz, CA). Cdk1/ckc2 (PSTAIR). The anti-PR antibodies used, AB-52 and B-30, were from our laboratories.

RESULTS

The Model System—PR immunoblots show that the two stable cell lines, T47D-YA and T47D-YB, contain equal amounts of PR-A or PR-B, respectively, and each isoform is expressed at levels comparable with its levels in the parental T47Dco cells (Fig. 1, *left panel*). These levels are half of the total PR in T47Dco. This was confirmed by ligand binding assays (not shown), in which the T47D-YA and T47D-YB cell extracts bind equivalent amounts of [^{3}H]R5020, at half the levels bound by T47Dco cell extracts. In addition, 6 h of progesterone generates the expected ligand-dependent phosphorylation and down-regulation (40) of both isoforms (Fig. 1, *right panel*) in a manner identical to that seen with wild-type T47Dco cells (not shown). This important ligand-dependent receptor down-regulation is required for strong transcriptional activity by progesterone (41).

Summary of Findings—To identify genes regulated by the two PR, replicate data points representing gene expression levels in T47D-YA or T47D-YB cells, and in PR-negative T47D-Y cells, treated with or without progesterone for 6 h, were analyzed by pairwise comparison. Genes that increased or decreased more than 1.8-fold in all three experiments and showed no significant variation among controls (PR negative cells, or triplicate "minus hormone" sets) were identified. Altogether, 94 genes of the 5,600 interrogated met these criteria (Table I). Fold changes are the average of triplicate experiments. In cases in which both receptors regulate the same gene, fold changes for each receptor are shown. Genes that were undetectable and called "absent" in one sample, but were detectable and called "present" in the other, are denoted with a tilde in Table I. (Note that the latter cannot be compared with fold changes for genes that were present in both samples, because the genes called absent were set to background levels.) All other genes represent ones that were present even in the absence of treatment but whose levels were altered by hormone. Genes indicated by Footnote a in Table I were identified with Atlas arrays; those indicated by Footnote c were identified using both Atlas and Affymetrix systems. All others were iden-

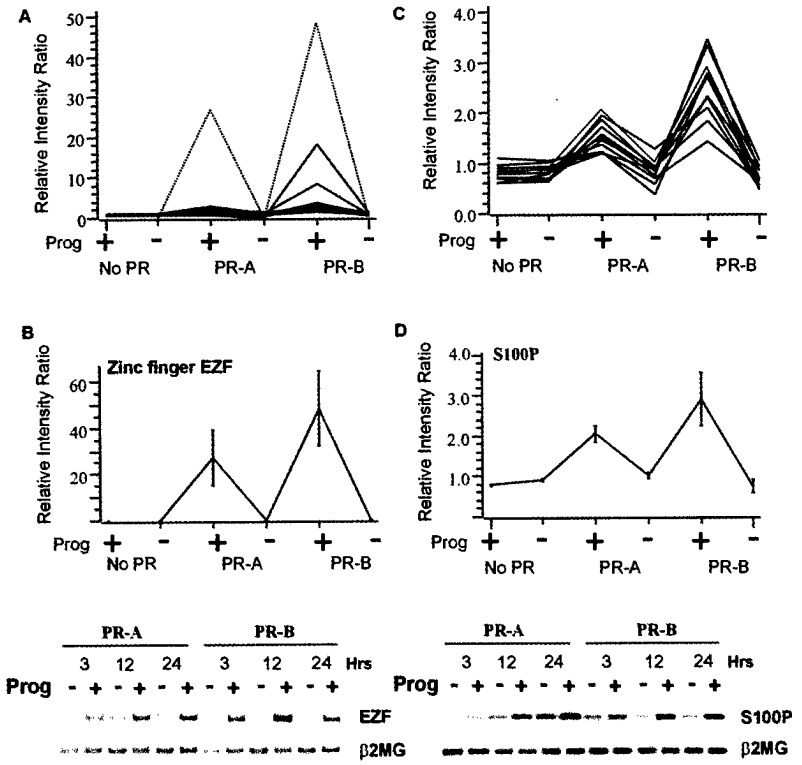

FIG. 2. **Genes up-regulated by progesterone through PR-A and PR-B.** Cells containing PR-A (T47D-YA cells), PR-B (T47D-YB cells), or no PR (T47D-Y cells) were treated with 10 nM progesterone (+) or ethanol vehicle (−) for 6 h. Labeled complementary RNA was generated and hybridized to Affymetrix HuGeneFL6800™ chips, as described under "Experimental Procedures." YA and YB cells were analyzed in triplicate, time-separated experiments, and Y cells were analyzed in duplicate experiments. Data were analyzed using MicroArray Suite 4.0 Expression Analysis Program™, and by GeneSpring™ version 4.0 performing *k*-means clustering. Statistical significance was assessed by one-way analysis of variance using a cut-off value of $p < 0.05$, followed by a Tukey multiple comparison test. Average relative intensity ratios indicate the relative expression levels of each gene in replicate experiments. All genes and average fold inductions are identified in Table I. *A*, cluster of genes up-regulated by progesterone in PR-A- and PR-B-containing cells but not in the PR-negative cells. A highly progesterone-regulated gene, the leucine zipper transcription factor, *EZF*, is shown as a *dashed line*. *B*, gene expression pattern of *EZF* isolated from the cluster in *A*, showing standard *error bars* for replicate experiments (*top*). RT-PCR using cDNA generated from T47D-YA and -YB cells treated with 10 nM progesterone (+) or vehicle (−) for 3, 12, and 24 h. Products were generated by RT-PCR using primers specific for *EZF* and *β2MG*. *C*, because the average difference value for each gene was normalized to itself by dividing all measurements for that gene by the median of the expression values of the gene over all samples, genes expressed at detectable levels in all samples have relative intensity ratios that cluster around 1.0. These genes are shown in a re-scaled version of *A*. *D*, gene expression pattern for *S100P* isolated from *C*, showing standard *error bars* for replicate experiments (*top*). RT-PCR from cDNA from T47D-YA or -YB cells treated with 10 nM progesterone (*Prog*) (+) or ethanol vehicle (−) for 3, 12, and 24 h was performed with specific primers for *S100P* and *β2MG*.

tified with the Affymetrix chips.

In summary, there are six sets of progesterone-regulated genes as follows: (i) 59 genes uniquely up-regulated by PR-B; (ii) 4 uniquely up-regulated by PR-A; (iii) 19 up-regulated by both receptors; (iv) 6 uniquely down-regulated by PR-B; (v) 0 uniquely down-regulated by PR-A; and (vi) 6 down-regulated by both receptors. These data demonstrate that the two PR isoforms largely regulate different subsets of genes. The low number of genes regulated by both receptors was a surprising outcome. Based on progesterone-induced fold changes in gene expression levels in the presence of cycloheximide, over half of the progesterone-regulated genes (51 of 94) are direct targets of PR. These are indicated as Footnote b after the accession number in Table I.

Functional Categories, Known Progesterone-regulated Genes, and Breast Cancer / Mammary Gland Development Genes—The genes were organized into functional categories (Table I) based on GeneCard information and an extensive review of the literature. Categories containing multiple progesterone-regulated genes include the following: (i) a large number of membrane-associated proteins including cell adhesion and cytoskeletal proteins, cytokines, and cytokine receptors, chemokines, secreted proteins, calcium-binding proteins, and membrane signaling molecules; (ii) many steroid, lipid, and general metabolic proteins; (iii) nucleic acid and protein processing factors; (iv) proteins involved in cell growth and apoptosis; and (v) transcription factors. Together these genes draw a picture of progesterone as an important metabolic hormone, with many surprising cell surface effects.

Sixteen of the 94 genes found to be regulated by progesterone in the present study have been reported previously to be progesterone-responsive in either breast cancer cells or other hormone-responsive cell types or tissues (Table II). The independent confirmation of these 16 genes serves as an internal control and demonstrates the quality of our data. The data described here increase the number of known progesterone-regulated genes by ~6-fold. A set of 10 genes had been reported previously to be involved in either breast cancer or mammary gland development (Table III). Most, however, were not previously known to be progesterone-regulated.

Cluster Analysis and Confirmatory Studies—Average differences indicating relative intensities from replicate data sets were entered into GeneSpring™. Gene expression measurements were scaled so that they could be plotted on a reasonable *y* axis for clustergram visualization. This was accomplished by normalizing each gene to itself (by dividing all measurements for each gene by the median of its expression values across all samples). Normalized values below 0 were set to 0. Because of these normalization procedures, the fold changes may appear different in the clustergrams than reported in Table I using

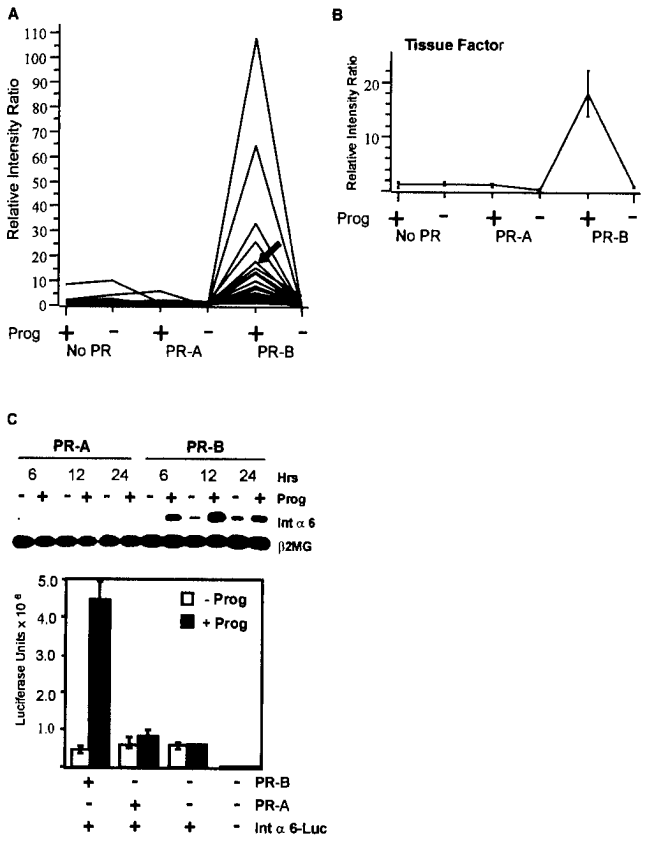

FIG. 3. **Genes uniquely regulated by progesterone through PR-B.** *A*, expression cluster generated as described for Fig. 2, showing genes significantly up-regulated by 6 h of progesterone (*Prog*) treatment only in the PR-B-expressing T47D-YB cells. The gene encoding tissue factor is indicated with an *arrow*. *B*, *top*, gene expression profile of the tissue factor transcript isolated from *A*, showing standard *error bars* for replicate experiments. *Bottom*, autoradiographic image of [α-^{32}P]dCTP incorporated into RT-PCR products generated using primers specific for tissue factor (*TF*) and *β2MG*, using cDNA generated from T47D-YA and -YB cells treated with 10 nM progesterone (+) or vehicle (−) for 3, 12, and 24 h. *C*, *top*, [α-^{32}P]dCTP-labeled products were generated by RT-PCR using primers specific for integrin α_6 and *β2MG*, using cDNA isolated from T47D-YA and -YB cells treated with 10 nM progesterone (+) or vehicle (−) for 6, 12, and 24 h. *Bottom*, HeLa cells transiently transfected with, from *left* to *right*, a promoter for integrin α_6 linked to a luciferase reporter (pGL3 vector) and either PR-B or PR-A expressed in pSG5, the empty pSG5 vector with the integrin α_6 promoter, or the empty pSG5 and empty pGL3 vectors, in triplicate dishes. Cells were treated with ethanol vehicle (*open bars*) or 10 nM progesterone (*solid bars*) for 24 h and harvested. Relative luciferase activity units corrected for transfection efficiency using the β-galactosidase expression plasmid pCH110 are shown with standard deviations for triplicate determinations.

Affymetrix algorithms. Within clusters, any one gene can be viewed individually, and standard error bars generated from the replicate experiments are then shown. Only genes regulated in a statistically significant manner are listed in Table I, and although clusters were generated by *k*-means, only those genes that were regulated in a statistically significant manner were left in the clusters. For several genes of interest, the array data were confirmed by measurement of the expressed transcripts by RT-PCR, or the proteins by Western blotting, to assess progesterone regulation at multiple time points. Additionally, for two differentially regulated genes, *ITGA6* and *BCL-X*, the isoform specificity of the regulation was confirmed by *in vitro* transcription assays using their promoters, in a cell line other than T47D.

PR-A and PR-B Up-regulated Genes—Nineteen genes were up-regulated by both PR isoforms. Fig. 2*A* shows a cluster of such genes. They are up-regulated by progesterone in both PR-A and PR-B containing cells but not in the PR-negative cell line. The gene encoding the leucine zipper protein, *EZF*, shown as a *dashed line* in Fig. 2*A* and is isolated in Fig. 2*B*, *top*, to show the standard error for triplicate determinations. *EZF* was below detectable levels on the Affymetrix chips in all cell lines in the absence of progesterone and was detectable only in PR-positive cells in the presence of progesterone. *EZF* was detectable at 25 cycles by RT-PCR, however, and was up-regulated after progesterone treatment at 3, 12, and 24 h in the presence of both PR-A and PR-B (Fig. 2*B*, *bottom*).

Most genes were expressed at detectable basal levels, even in the absence of progesterone, and for such genes, dividing by the mean over all samples results in relative intensity ratios of ~1.0, as shown when the data from Fig. 2*A* are re-scaled in Fig. 2*C*. Although some of the genes in Fig. 2*A* were more strongly up-regulated by PR-B than PR-A, others, as shown in Fig. 2*C*, are equally well regulated by both PR isoforms. An example of the latter is the gene encoding calcium-binding protein *S100P* (Fig. 2*C*, in *red*). *S100P* is up-regulated by both PR (Fig. 2*D*, *top*) and is up-regulated as early as 3 h and remains elevated after 24 h of progesterone treatment, as shown by RT-PCR (Fig. 2*D*, *bottom*).

PR-B Up-regulated Genes—The majority of genes (59 of 94) are uniquely up-regulated by PR-B as illustrated by the cluster in Fig. 3*A* and in Table I. Tissue factor (*F3*), indicated by an *arrow* in Fig. 3*A* and isolated in Fig. 3*B*, *top*, is one example. Its differential regulation by PR-B was confirmed by RT-PCR (Fig. 3*B*, *bottom*). The tissue factor transcript is up-regulated 3 and 12 h after the start of progesterone treatment but only in cells expressing PR-B. It is undetectable 24 h after the start of progesterone, however, indicating that its regulation is transient.

Integrin α_6 is also regulated only by PR-B. This was observed using Atlas™ arrays and was confirmed by RT-PCR at 6, 12, and 24 h after progesterone treatment (Fig. 3*C*, *top*). The integrin α_6 promoter had been cloned and reported to be progesterone-responsive (42). We therefore used this promoter, linked to luciferase, to demonstrate the PR isoform specificity of its regulation in an exogenous transcription system and a different cell line (Fig. 3*C*, *bottom*). Indeed, the integrin α_6 promoter was induced 9-fold by progesterone in HeLa cells transfected with PR-B but was not induced by PR-A. That the differential regulation of this promoter was recapitulated in an entirely different cell line and system validates the PR-B-specific regulation in T47D cells and provides a model where the mechanisms underlying the isoform specificity can be dissected.

STAT5A and C/EBPβ are two important mammary gland regulatory proteins (43–45). Their expression levels are also controlled only by PR-B (Fig. 4). Fig. 4*A* shows regulation of Stat5a by PR-B isolated from the clustergram shown in Fig. 3*A*. Its preferential regulation by PR-B is confirmed by the immunoblot in Fig. 4*B* (*black arrow*). In the same experiment, p21 and cyclin D1, known progesterone-regulated genes (46–48), are equally well regulated by either PR isoform (Fig. 4*B*).

Gene array data for *C/EBPβ* regulation by progesterone are shown in Fig. 4*C*. The protein product of this gene is also uniquely regulated through PR-B as confirmed by the immunoblot (Fig. 4*D*), using antibody specific for the Lap isoforms of C/EBPβ. The C/EBPβ Lip isoforms are also up-regulated by progesterone only through PR-B (not shown). Again, cyclin D1 and p21 are regulated by both PR isoforms. Note that cyclin D1 is up-regulated at 24 h (Fig. 4, *B* and *D*), but returns to control by 48 h (Fig. 4*D*). In contrast, p21 is still elevated at 48 h.

PR-A Up-regulated Genes—Only four genes were preferentially up-regulated by PR-A (Fig. 5 and Table I). The gene encoding the docking protein, enhancer of filamentation

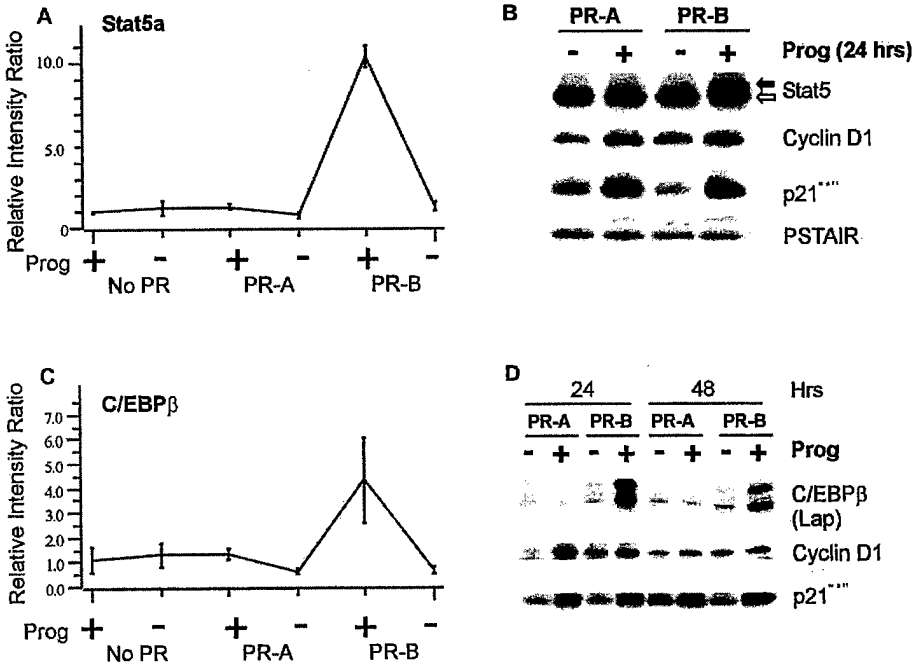

FIG. 4. **STAT5A and C/EBPβ are uniquely up-regulated by progesterone only through PR-B.** *A*, cells were treated and analyzed as described in Fig. 2. The gene expression pattern of Stat5a (isolated from the clustergram in Fig. 3*A*) is shown to demonstrate standard error bars generated from replicate experiments. *B*, T47D-YA and -YB cells were treated with 10 nM progesterone (*Prog*) (+) or ethanol vehicle (−) for 24 h. Cells were harvested, and 100 μg of whole cell lysates were resolved by SDS-PAGE and immunoblotted with AB-52 antibody, which recognizes both isoforms of PR, with antibody to total Stat5, which recognizes both STAT5A (*solid arrow*) and −5b (*open arrow*), or with antibodies to cyclin D1, $p21^{WAF1}$, or to PSTAIR. *C*, the gene expression pattern of C/EBPβ isolated from the clustergram in Fig. 3*A*, showing standard errors. *D*, T47D-YA and -YB cells were treated with 10 nM progesterone (+) or ethanol vehicle (−) for 24 and 48 h, harvested as described above, and immunoblotted with antibodies that recognize C/EBPβ lap isoforms, cyclin D1, or $p21^{WAF1}$.

(*HEF1*), is predominantly up-regulated by PR-A (Fig. 5*A*) as shown by the array data (*top*) and RT-PCR data (*bottom*). The gene encoding the orphan nuclear receptor, estrogen-related receptor α (*ERRα*), also is only significantly up-regulated by PR-A (Fig. 5*B*), as shown by the array data (*top*) and RT-PCR (*bottom*). Finally, the anti-apoptosis gene *BCL-X_L* appears to be uniquely up-regulated by PR-A. This was first observed with the Atlas™ macroarrays (not shown). The Affymetrix microarray data were equivocal (Fig. 5*C*, *top*), as standard error bars were large. However, RT-PCR (Fig. 5*C*, *bottom*) clearly demonstrated preferential regulation by PR-A. To confirm the unique regulation of *BCL-X_L* by PR-A, we isolated ~1000 bp of the human *BCL-X* promoter and cloned it in front of a luciferase reporter (Fig. 5*D*). The construct was transfected into HeLa cells together with one or the other PR isoform, and cells were treated with or without progesterone. Hormone-dependent regulation of the *BCL-X* promoter was observed only in the presence of PR-A (Fig. 5*D*).

Down-regulated Genes—In general, progesterone-induced gene down-regulation was uncommon, but 12 such genes were identified (Table I) by pairwise comparison of triplicate experiments using MicroArray Suite. Additionally, gene filtering using GeneSpring™ generated a clustergram of genes regulated in this manner (Fig. 6*A*). Progesterone-mediated down-regulation of two of these genes (*highlighted* in *red* in Fig. 6*A*), monocyte chemotactic protein (*MCP*; *open arrow* in Fig. 6*A* and isolated in Fig. 6*B*, *top*) and bullous pemphigoid antigen (*BPAG*; *closed arrow* in Fig. 6*A* and isolated in Fig. 6*B*, *bottom*), was confirmed by RT-PCR, particularly at early time points (Fig. 6*C*). This down-regulation is in contrast to the gene encoding 11β-hydroxysteroid dehydrogenase (*HSD11β2*), which is up-regulated by progesterone in the same RT-PCR experiment (Fig. 6*C*). *β2MG* served as a loading control.

DISCUSSION

Overview—This study, the first global examination of PR regulated genes in any system, reveals the molecular basis for functional differences between the two PR isoforms. We demonstrate that in breast cancer cells, although some genes are regulated by progesterone through both PR isoforms, most genes are uniquely regulated through one or the other isoform and predominantly through PR-B. These studies were performed in homogenous tumor cell populations allowing quantitative, statistical analyses of replicate independent experiments and straightforward interpretation of the data. This is difficult to do in organs or tumors that contain mixed cell types, without or with malignant epithelium. The results are validated by recent studies (49) that classified cell lines from various types of cancers based on their gene expression patterns, and found strong correlations among cell lines, the primary tumors from which they were derived, and the normal tissue of origin. This indicates that adaptation for growth in culture does not overwrite the gene expression programs established during tissue differentiation *in vivo*. Thus, our findings in T47D cells regarding progesterone-mediated gene regulation will apply to other breast cancer cells and normal progesterone target tissues. Sixteen of 94 progesterone responsive genes identified here have been reported to be progesterone regulated in other tissues and models.

Practical Applications and PR Measurements in Breast Cancer—An excess of one or the other PR isoforms may result in tumors with different prognostic and hormone-responsiveness profiles than tumors that have equimolar levels of the two PR. If so, it would be clinically important to distinguish among these tumor subsets. Current immunohistochemical clinical PR assays are incapable of doing this. In fact, it has been discovered recently that several anti-PR antibodies used clinically fail to detect PR-B by immunohistochemistry, even if they can do so by immunoblotting (50). Therefore, current clinical assays may fail to measure what may be the biologically more active PR isoform in breast cancers (PR-B) and do not distinguish between the two isoforms. It is possible that in the future judicious selection, and measurement, of progesterone-regulated genes can substitute for measurement of the receptors.

The data can also serve as the standard against which future studies of progesterone action in other cell types and tissues will be compared. This may provide explanations for differences in function of the two PR isoforms in different tissues. The preponderance of genes regulated uniquely by PR-B in breast cancer cells is surprising. Until progesterone-regulated gene expression profiles are reported in other cells and tissues, we will not know whether this dominance by PR-B is, or is not, unique to breast cancer. Recall that in PR-A knockout mice, the

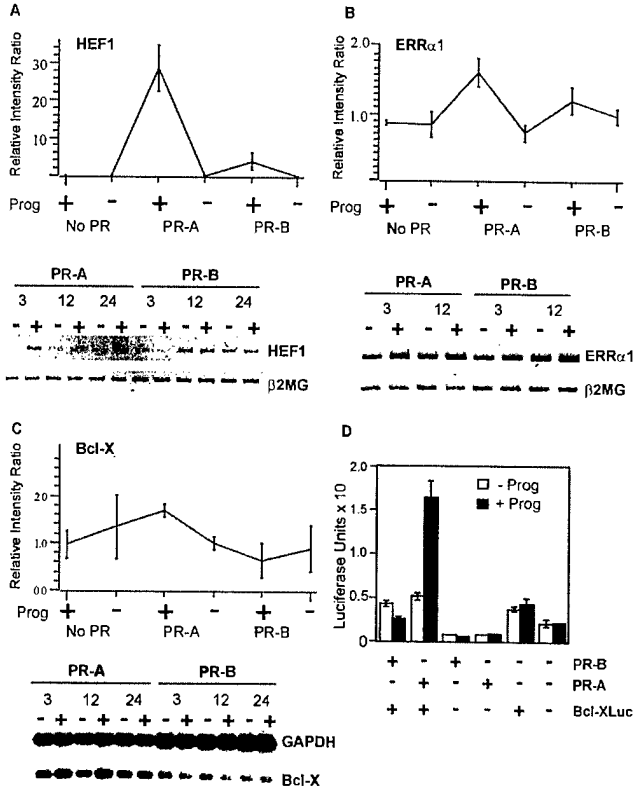

FIG. 5. **Genes uniquely regulated by progesterone through PR-A.** *A*, *top*, the expression profile of the isolated enhancer of filamentation 1 gene (*HEF-1*) is shown including standard *error bars* for triplicate determinations. *Bottom*, reverse image of ethidium bromide-stained RT-PCR products generated using primers specific for *HEF-1* and *β2MG* from cDNA isolated from T47D-YA and -YB cells treated with 10 nM progesterone (*Prog*) (+) or vehicle (−) for 3, 12, and 24 h. *B*, *top*, isolated gene expression pattern of estrogen-related receptor (*ERRα1*). *Bottom*, RT-PCR performed with primers specific for ERRα1 and β2MG from cDNA prepared from T47D-YA and -YB cells treated with 10 nM progesterone (+) or vehicle (−) for 3, and 12 h. *C*, *top*, isolated gene expression pattern of *BCL-X$_L$*. *Bottom*, [α-^{32}P]dCTP-labeled products generated by RT-PCR performed with primers specific for *BCL-X$_L$* and *GAPDH* from cDNA isolated from T47D-YA and YB cells treated with 10 nM progesterone (+) or vehicle (−) for 3, 12, and 24 h. *D*, HeLa cells were transiently transfected with the following constructs from *left to right*: a promoter for *BCL-X$_L$* linked to the luciferase reporter (pA3Luc) isolated as described under "Experimental Procedures," together with either PR-B or PR-A expressed in pSG5; PR-B or PR-A with empty pA3LucLink; empty pSG5 with the *BCL-X$_L$* promoter; or empty pSG5 and no promoter, in triplicate dishes. Cells were treated with ethanol vehicle (*open bars*) or 10 nM progesterone (*solid red bars*) for 24 h and harvested. Relative luciferase activity units normalized to β-galactosidase expression plasmid pCH110 are shown with standard deviations for triplicate determinations.

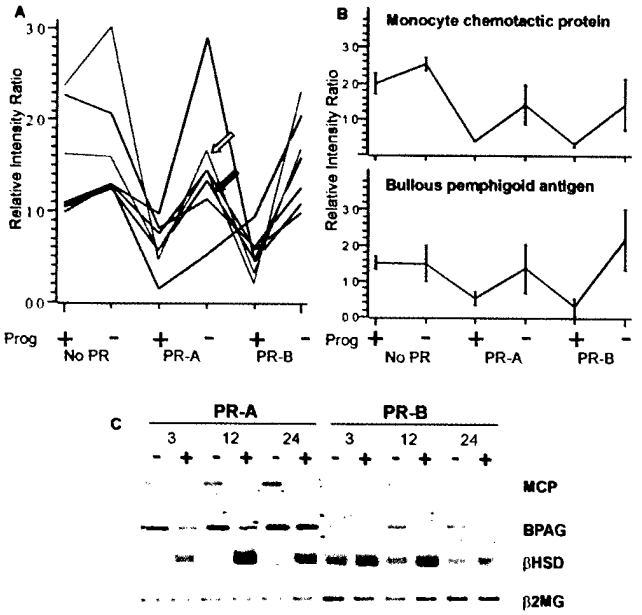

FIG. 6. **Some genes are down-regulated by progesterone.** *A*, a cluster of genes down-regulated by progesterone in cells containing either PR-A or PR-B, but not in PR-negative cells. Two genes for which confirmatory data are shown, are highlighted in *red*. The *open arrow* indicates monocyte chemotactic protein (*MCP*) and *closed arrow* indicates bullous pemphigoid antigen (*BPAG*). *B*, *top*, isolated expression pattern for the gene encoding MCP. *Bottom*, isolated expression pattern for the gene encoding BPAG. *C*, reverse image of ethidium bromide-stained RT-PCR products generated using primers specific for *MCP*, *BPAG*, *HSD11β2* (an upregulated gene, shown in contrast), and *β2MG*, using cDNA isolated from T47D-YA and -YB cells treated with 10 nM progesterone (+) or vehicle (−) for 3, 12, and 24 h.

virgin mammary gland develops normally, but the reproductive tract exhibits hyperproliferative anomalies consistent with failure of progesterone to oppose the actions of estrogens when only PR-B are present (4). It is possible that PR-A have a more important ER repressor function in the endometrium than they do in the breast. If so, this may be reflected in a different gene expression profile for PR-A in endometrial cells. Interestingly, we observe that *ERRα1* levels are preferentially up-regulated by PR-A (Fig. 5*B*). Because ERRα1 can regulate some of the same target genes as ERα and interfere with the functional activity of ERα (51), this may be a molecular mechanism by which PR-A modulate the activity of ERα *in vivo*.

Genes Regulated in the Normal Breast and Breast Cancers—We show that STAT5A, MSX-2, and C/EBPβ are up-regulated only by PR-B (Table I and Fig. 4). The PR-B-specific regulation of these three proteins, known to be critical for normal mammary gland development (43–45, 52, 53), is consistent with data demonstrating that the mammary gland develops normally in PR-A knockout mice that contain only PR-B (4).

The following genes, newly found to be progesterone-regulated (Table I), are differentially expressed in breast cancer compared with normal breast (Table III). 1) Bullous pemphigoid antigen (*BPAG*) is down-regulated by progesterone through both PR isoforms. The protein, associated with hemidesmosome formation, is 12-fold overexpressed in normal compared with malignant breast epithelium (54). In the normal breast it may be involved in the regulation of milk secretion (55). Expression of hemidesmosome component proteins is lost in invasive breast and other cancers (56, 57). We suggest that this deleterious effect may be exacerbated by progesterone. 2) Expression of the gene encoding calcium-binding protein S100P is up-regulated by progesterone through both isoforms. Overexpression of S100P is associated with immortalization of human breast epithelial cells *in vitro* and with early stage breast cancer development *in vivo* (58). Progesterone would exacerbate this deleterious effect. 3) The gene encoding EZF, a zinc finger transcription factor, is up-regulated by progesterone through both isoforms. EZF is up-regulated during breast cancer progression (59). Progesterone would exacerbate this deleterious effect. 4) The gene encoding tissue factor, a cell surface glycoprotein, is up-regulated by progesterone uniquely through PR-B. Tissue factor is associated with metastasis in breast and other cancers (60, 61) and is regulated by progesterone in the endometrium during decidualization (62–64). Its up-regulation by progesterone in breast cancers might enhance metastasis. 5) The gene encoding GAS6, a ligand for the tyrosine kinase receptor, Axl receptor tyrosine kinase, is also uniquely regulated by PR-B. GAS6 is mitogenic in breast cancer cells (65) and promotes chemotaxis of vascular smooth muscle cells (66). Its up-regulation by progesterone in breast cancers might be deleterious. 6) The anti-apoptosis gene, *BCL-X$_L$*, is up-regulated

only by PR-A. Resistance to apoptosis by preferential up-regulation of BCL-X_L could explain the deleterious effect of PR-A overexpression in the mammary gland of transgenic mice (31). 7) HEF1, a docking protein associated with focal adhesion kinase (67), is also preferentially up-regulated by PR-A. HEF1 is related to BCAR1/p130^{Cas}, which is up-regulated in tamoxifen-resistant tumors (68, 69). Are tumors overexpressing PR-A more resistant to apoptosis-inducing chemotherapeutic agents or to tamoxifen? Taken together, our data raise the possibility that physiological progesterone levels are harmful in breast cancer, and may explain recent HRT data that, unlike its effect in the uterus, progesterone is not protective in the breast and indeed increases breast cancer risk (11, 12).

Progesterone and the Cell Membrane—The genes that we have discovered to be progesterone-regulated are involved in particular functional pathways as shown in Table I. It was previously known, for example, that progesterone regulates proteins involved in steroid biosynthesis and trafficking pathways (70–72), so our confirmation of this role for the hormone is not surprising. However, the extensive number of genes involved in membrane-initiated events that we define as being progesterone-regulated is surprising (Table I). These include proteins involved in cell adhesion, membrane receptors, calcium-binding proteins, and signaling molecules including genes involved in G protein signaling. Together they represent almost half of all progesterone-regulated genes. These data clearly point to the membrane as an important target of progesterone action.

Mechanisms—Most normal progesterone target cells express both PR-A and PR-B. The studies described here define the gene regulatory properties of each isoform independently. This information is critical to understanding the more complex question: how the presence of one isoform influences gene regulation by the other. Our preliminary data (not shown), using T47Dco cells that contain both receptors, suggest that presence of PR-A can suppress up-regulation of some but not all PR-B-specific genes. For example, transcripts for *GAS6* and *STAT5A* are up-regulated 9.3- and 6.1-fold, respectively, in PR-B containing T47D-YB cells, but their levels are unaffected by progesterone in T47Dco cells. This suggests that in the T47Dco cells, PR-A suppress the effects of PR-B on these genes. Other genes, for example *C/EBPβ* and the zinc finger transcription factor, *AREB6*, are up-regulated in both T47D-YB and T47Dco cells. Clearly, presence of PR-A does not suppress expression of these PR-B-specific genes in T47Dco cells. The underlying mechanisms for these differences will require studies of the specific gene promoters. To that end, we are isolating key promoters. We have also generated new inducible cell lines, in which the expression of each isoform as well as the PR-A to PR-B ratio can be controlled. These cells are also being used to confirm the apparent ligand-independent PR regulation of some genes.

Interestingly, the converse may be simpler. Genes up-regulated only by PR-A (Fig. 5), such as *BCL-X_L* and *ERRα1*, are also up-regulated in T47Dco cells, suggesting that PR-B lack the inhibitory properties of PR-A. We hypothesize that genes regulated only by PR-B require the AF3 function unique to PR-B. This would further suggest that genes regulated by both PR isoforms do not require AF3. If so, there might be three subsets of progesterone-regulated genes as follows: those regulated by the PR-B homodimer, those regulated by the PR-A homodimer, and those regulated by the PR heterodimer. We are now in position to test these ideas using the inducible cell lines and mutant PR-B that lack AF3 activity (73).

Concluding Remarks—The actions of progesterone in the breast are paradoxical because the hormone has both proliferative and differentiative functions therein. This is in apparent contrast to the uterus, where its actions are mainly antiproliferative. Therefore, to protect the uterus, progestins are often added to estrogens for HRT. However, this regimen is controversial, because recent evidence suggests that the progestins in HRT increase the risk of breast cancer (11, 12). Given that expression of one or the other PR isoform may be more or less beneficial in certain physiological states or tumors, it would be useful to have ligands that activate or suppress one of the isoforms preferentially. By using specific subsets of the genes we have identified here, together with cell lines that express only one or the other PR isoform, one can screen large libraries of candidate progestins and antiprogestins for isoform specificity. Along those lines we can now ask how gene regulation profiles compare when the ligand is progesterone, or one of the many synthetic progestins in widespread clinical use such as medroxyprogesterone acetate.

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Appendix B

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New Human Breast Cancer Cells to Study Progesterone Receptor Isoform Ratio Effects and Ligand-independent Gene Regulation*

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AQ: A

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All known progesterone target cells coexpress two functionally different progesterone receptor (PR) isoforms: 120-kDa B-receptors (PR-B) and N-terminally truncated, 94-kDa A-receptors (PR-A). Their ratio varies in normal and malignant tissues. In human breast cancer cells, homodimers of progesterone-occupied PR-A or PR-B regulate different gene subsets. To study PR homo- and heterodimers, we constructed breast cancer cell lines in which isoform expression is controlled by an inducible system. PR-negative cells or cells that stably express one or the other isoform were used to construct five sets of cells: (i) PR-negative control cells (Y iNull), (ii) inducible PR-A cells (Y iA), (iii) inducible PR-B cells (Y iB), (iv) stable PR-B plus inducible PR-A cells (B iA), and (v) stable PR-A plus inducible PR-B cells (A iB). Expression levels of each isoform and/or the PR-A/PR-B ratios could be tightly controlled by the dose of inducer as demonstrated by immunoblotting and transcription AQ: B **studies. Induced PRs underwent normal progestin-dependent phosphorylation and down-regulation and regulated exogenous promoters as well as endogenous gene expression. Transcription of exogenous promoters was dependent on the PR-A/PR-B ratio, whereas transcription of endogenous genes was more complex. Finally, we have described several genes that are regulated by in-** AQ: C **duced PR-A even in the absence of ligand.**

Progesterone exerts its effects through progesterone receptors (PRs),[1] which are ligand-dependent members of the nuclear receptor family of transcription factors. Two PR isoforms exist in progesterone target tissues: the 120-kDa B-isoform (PR-B) and the N-terminally truncated, 94-kDa A-isoform (PR-A). In transient transfection systems, the two receptors have markedly different transcriptional effects (1–3). Antiprogestins have partial agonist effects only on PR-B, whereas PR-A functions as a repressor (1, 3, 4). Differential regulation by the two PR isoforms occurs on endogenous genes as well. Microarray analyses demonstrate that the two PRs up- and down-regulate different subsets of genes in breast cancer cells (5). For example, although the genes encoding the cell cycle regulatory proteins p21 and cyclin D1 are equally well up-regulated by both receptors (6), the anti-apoptotic gene *bcl-x*$_L$ is uniquely up-regulated by PR-A, whereas PR-B uniquely up-regulates CAAT/enhancer-binding protein-β, *STAT5α*, integrin α_6 (*ITGA6*), and tissue factor F3 (5), all genes important for mammary gland growth, differentiation, and/or breast cancer. AQ: D

An additional complexity arises from the fact that the two isoforms are coexpressed in the same cell. Therefore, the function of PR-A/PR-B heterodimers may differ from that of the homodimers. Because of this, the ratio of PR-A to PR-B in a tissue is likely to control its response to progesterone. In the uterus, PR-A/PR-B ratios vary extensively during the menstrual cycle (7, 8), leading to variable progesterone responsiveness. PR knockout mice and transgenic mice that overexpress one PR isoform demonstrate the importance of a balanced isoform ratio. Mice that express only PR-B exhibit normal mammary gland development, but have severe reproductive tract anomalies (9), indicating that isoform expression defects are tissue-specific. Transgenic mice that overexpress PR-A exhibit abnormal mammary gland development, including ductal hyperplasia, extensive ductal branching, and decreased cell-to-cell adhesion, all features associated with neoplasia (10). In contrast, overexpression of PR-B reduces ductal branching and alveolar development (11). Taken together, the data suggest that PR-A and PR-B have physiologically different tissue-specific functions and that maintenance of appropriate isoform ratios is required for normal progesterone responses.

The two PRs are expressed at equimolar levels in the normal human breast during the menstrual cycle (12). Whether the PR ratio fluctuates during development or pregnancy is unknown. In human breast cancers, measurement of total PR levels is an important guide to disease prognosis and response to hormone therapies (13, 14). However, the role of each isoform in clinical decision-making is unknown, but three studies have addressed this (12, 15, 16). Immunoblot analyses of 202 PR-positive breast cancers show that in ~50% of tumors, one isoform exceeds the other (12, 16). Among such invasive tumors, PR-A predominates in ~80% of cases (12). Tumors that overexpress PR-A are less differentiated than tumors that overexpress PR-B (15). Interestingly, in culture, human breast cancer cells that overexpress PR-A detach from the monolayer in response to progesterone (17), a phenotype associated with high-grade malignancies.

In PR-positive T47Dco human breast cancer cells, the two isoforms are constitutively expressed at equimolar levels (18, 19). These cells are ideal models to study progesterone action

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[1] The abbreviations used are: PRs, progesterone receptors; PR-A, progesterone receptor A-isoform; PR-B, progesterone receptor B-isoform; STAT, signal transducer and activator of transcription; ponA, ponasterone A; PRLR, prolactin receptor; MEM, minimum Eagle's medium; CSS, charcoal-stripped serum; MMTV, murine mammary tumor virus; Luc, luciferase; PRE, progesterone response element; WCEs, whole cell extracts; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

because PR induction does not require estrogen pretreatment. In previous studies, a PR-negative subline (T47D-Y cells) was isolated from T47Dco cells and used to stably reintroduce constitutively expressed PR-A (T47D-YA cells) or PR-B (T47D-YB cells) (20). Using the ecdysone-inducible system (21), we have now used these cells to engineer five new cell lines. T47D-Y cells were used to create cells that inducibly express (i) no PR (Y iNull cells), (ii) PR-A (Y iA cells), or (iii) PR-B (Y iB cells). Additionally, to manipulate isoform ratios, (iv) T47D-YA cells were modified to inducibly express PR-B (A iB cells), and (v) T47D-YB cells were modified to inducibly express PR-A (B iA cells). The five cell lines all have the same parental cell background. Four express ponasterone A (ponA)-inducible PRs in a tightly regulated manner; and in the cells that express one isoform constitutively, the PR-A/PR-B ratio can be controlled by induction of the other isoform. We demonstrate that liganded, induced receptors undergo progesterone-dependent phosphorylation and down-regulation and control exogenous progesterone-responsive promoters and endogenous gene transcription. A novel finding is that upon receptor induction, subsets of genes defined by microarrays are regulated even in the absence of ligand. One of these genes encodes prolactin receptors (PRLRs).

EXPERIMENTAL PROCEDURES

Cell Lines and Culture—The PR-positive T47Dco breast cancer cell line, isolation of its PR-negative clonal derivative T47D-Y, and construction of PR-positive T47D-YA and T47D-YB cells have been described (20, 22). Cells were routinely cultured in 75-cm^2 plastic flasks and incubated in 5% CO_2 at 37° C in a humidified environment. The stock medium consisted of minimum Eagle's medium (MEM) with Earle's salts containing L-glutamine (292 μg/liter) buffered with sodium bicarbonate (2.2 μg/liter), insulin (6 ng/ml), and 5% fetal calf serum (Hyclone Laboratories, Logan, UT). The T47D-YA and T47D-YB cells were grown in 200 μg/ml G418 (Sigma). The Y iNull, Y iA, Y iB, A iB, and B iA cells were maintained in medium as described above with 300 μg/ml Zeocin (Invitrogen) and 145 units/ml hygromycin B (Calbiochem). A iB and B iA cells were also maintained in 200 μg/ml G418.

Plasmid Construction—The ecdysone-inducible mammalian expression plasmids, and the VgRXR, pInd/hygro, and Ind/LacZ plasmids were from Invitrogen. Five μg of the pInd/hygro vector containing the hygromycin B resistance gene were digested with *Eco*RV and dephosphorylated. The PR-B cDNA (human PR1, gift of P. Chambon, Strasbourg, France) (23) was released with *Eco*RI, and its ends were filled with Klenow and ligated to the *Eco*RV-digested pInd/hygro vector to generate IndB/hygro. IndA/hygro was created by *Bam*HI digestion of IndB/hygro to excise the 5′-PR-B cDNA region (BUS), followed by religation. DNAs were sequenced for orientation and content.

T47D Cell Transfection and Selection of Stable Cell Lines—Approximately 3 million T47D-Y, T47D-YA, or T47D-YB cells were transfected with 15 μg of the VgRXR plasmid. After 48 h, the cells were placed in medium containing 500 μg/ml Zeocin to kill untransfected cells. Positive clones were expanded and tested for VgRXR expression and function by β-galactosidase expression from the transiently transfected Ind/LacZ vector containing five VgRXR-binding sites (E/GREs) upstream of the LacZ reporter. Cells were induced for 24–48 h with 10 μM ponA (Invitrogen) and lysed in 1× lysis buffer (Pharmingen, San Diego, CA), and β-galactosidase assays were performed as described (6). Three clones with the highest induction (T47D-YV, T47D-YAV, and T47D-YBV) were selected and used to generate secondary stable cells. T47D-YV cells were transfected by electroporation with 15 μg of the IndA/hygro, IndB/hygro, or IndNull/hygro construct; T47D-YAV cells were transfected with IndB/hygro; and T47D-YBV cells were transfected with IndA/hygro. After 48 h, cells were placed in medium containing hygromycin B (195 units/ml). Surviving clones were expanded and assayed for PR expression by immunoblotting after 24 or 48 h of induction with 10 μM ponA. IndNull/hygro (Y iNull) cells were screened for an intact inducible promoter region by PCR (data not shown).

Transcription Assays—Cells were harvested, washed with phosphate-buffered saline, and resuspended in medium containing 6% charcoal-stripped serum (CSS). Four million cells, 12 μg of MMTV-Luc reporter (a gift of S. Nordeen, Denver, CO) (24) or PRE_2-$TATA_{tk}$-Luc, and 1 μg of cytomegalovirus-β-galactosidase as an internal control were electroporated at 220 V and 950 microfarads. Replicate sets were plated in four 35-mm dishes in MEM containing CSS, induced with 10 μM ponA or Me_2SO for 24 h, and then treated with EtOH or 10 nM R5020 for 24 h. Cells were harvested, washed with phosphate-buffered saline, and lysed with 1× lysis buffer. Luciferase and β-galactosidase assays were performed as described (6).

PR Immunoblotting—Whole cell extracts (WCEs) were prepared in radioimmune precipitation assay buffer with protease inhibitors as described (6) from cells that were induced or not for 24 h with 10 μM ponA. Extracts (200 μg) were resolved on a 7.5% denaturing polyacrylamide gel, transferred to nitrocellulose, blocked, and probed for PR with a mixture of AB-52 and B-30 antibodies (25). Protein bands were visualized by enhanced chemiluminescence (Amersham Biosciences) and quantified by densitometry (Alpha Imager, Alpha Innotech Corp., San Leandro, CA).

Time Course Studies—For PR induction, cells in log phase were harvested, and 1.3 million were plated in 60-mm dishes containing MEM and CSS with vehicle or 10 μM ponA. Cells were harvested at specified times (3–72 h), and WCEs were immunoblotted. For PR turnover, cells in log phase were plated at 1.3 million cells/60-mm plate and treated with Me_2SO or 10 μM ponA for 24 h. One of three treatments followed: 1) removal of ponA and addition of Me_2SO, 2) removal of ponA and addition of R5020, or 3) continued ponA and addition of R5020. Cells were harvested at the specified times, and WCEs were immunoblotted.

Reverse Transcription (RT)-PCR—Cells in log phase were changed into antibiotic-free MEM and 6% CSS containing Me_2SO or 10 μM ponA, induced for 24 h, and then treated with EtOH or progesterone (10 nM) for the specified times. Cells were harvested and washed, and total RNA was prepared. RT-PCR was performed using conditions as reported (5) with GAPDH as an internal control for each sample. PRLR primers were as follows: PRLRfwd, 5′-gcagctgagtgggagatcc-3′; and PRLRrev, 5′-ggacagccacagagatccac-3′. Other primer sequences were previously reported (5). Samples were resolved on 2% agarose gels and stained with ethidium bromide (reverse images are shown.) Densitometry was performed, and samples were normalized to GAPDH prior to calculation of -fold changes. Immunoblotting was performed to monitor PR induction.

Microarray Analysis—Y iA or Y iNull cells in log phase were changed into antibiotic-free MEM and 6% CSS containing Me_2SO or 10 μM ponA, induced for 24 h, and then treated with EtOH or progesterone (10 nM) for 6 h. Cells were harvested and washed, and total RNA was isolated. Poly(A)$^+$ RNA was prepared; samples were labeled; and microarray analysis was performed with Affymetrix gene chips (HuFL-U95Av2) as described (5). RNA samples were prepared from cells independently induced in three (Y iA) or two (Y iNull) time-separated experiments. Data were plotted (GeneSpring Version 4.0, Silicon Genetics, Redwood City, CA), normalized, and analyzed as previously described (5). An asterisk denotes a statistically significant difference as assessed by one-way analysis of variance using a $p < 0.05$ cutoff followed by a Tukey multiple comparison test between Me_2SO-treated (sets 1 and 2) and ponA-treated (sets 3 and 4) cells.

RESULTS

Construction and Description of Cells—The ecdysone-inducible mammalian expression system (21) was used to construct cells that inducibly express one or the other PR isoform in the background of PR-negative T47D-Y cells (20). The cells were stably transfected with the VgRXR plasmid, which encodes both the modified ecdysone receptor (Vg) and retinoid X receptor (RXR) regulatory proteins; selected in antibiotic; and expanded. Cells were transiently transfected with the inducible LacZ-positive control plasmid and induced with ponA, and the clone with the highest induction of β-galactosidase (termed T47D-YV) was stably transfected with plasmids containing five VgRXR-binding sites upstream of the PR-A (IndA/hygro) or PR-B (IndB/hygro) cDNA or no cDNA (pInd/hygro). Transfected cells were selected in antibiotic; expanded; induced with ponA; and screened by immunoblotting or RT-PCR to select clonal lines that inducibly express PR-A (Y iA cells), PR-B (Y iB cells), or no PR (Y iNull cells). Fig. 1 shows an immunoblot from the three cell lines treated either with vehicle or with ponA. PR regulation was tightly controlled, and PRs were undetectable in the uninduced cells and the control Y iNull cells. Multiple independent clones were characterized, and no significant dif-

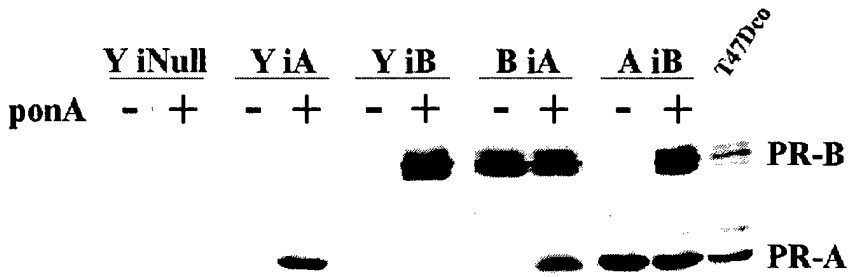

FIG. 1. **The cell lines inducibly express the desired PR isoform.** Cells were plated in medium containing Me_2SO or 10 μM ponA for 24 h and harvested, and immunoblotting was performed using 200 μg of WCEs. Western blotting was performed with a mixture of AB-52 and B-30 antibodies.

ferences were observed among them. Therefore, one representative clone for each inducible cell type is shown.

T47D-YA and T47D-YB cells, which constitutively express PR-A and PR-B, respectively, were transfected with the VgRXR plasmid; selected in antibiotic; expanded; and screened with the inducible LacZ construct. The highest expressers, called T47D-YAV and T47D-YBV, were used to create the secondary cells. YAV cells received the IndB construct; YBV cells received the IndA construct. Positive clones were selected in hygromycin B, expanded, and screened by immunoblotting (Fig. 1). In the absence of inducer, only the constitutive isoform was expressed. Upon addition of ponA, the second isoform appeared. Expression of the inducible isoform did not affect expression of the constitutive isoform, and receptor levels were similar to those found in T47Dco cells, which express both PR-A and PR-B naturally (Fig. 1).

PR Induction Is ponA Dose-dependent—Because heterodimerization of the VgRXR regulatory protein is dependent on binding of ponA, inducer concentration determines the amount of protein produced by the target gene. To demonstrate this, cells were treated with increasing concentrations of ponA (Fig. 2). PR induction was detectable with 1–3 μM ponA in Y iA and Y iB cells (Fig. 2*A*). In A iB cells, the PR-A/PR-B ratio ranged from 2.3 to 0.9 depending on the ponA dose (Fig. 2*B*). In B iA cells, the PR-A/PR-B ratio ranged from 0.3 (at 3 μM ponA) to 2.5 (at 10 μM ponA) (Fig. 2*B*). For the studies described below, a 10 μM ponA dose was used.

PR Induction Is Time-dependent—Cells were treated with vehicle or ponA for 3 to 72 h (Fig. 3). PR induction was readily observed at 12 h and peaked at 24 h (Fig. 3, *A* and *B*). Without addition of fresh ponA, PRs stayed at high levels for varying periods of time: 72 h in Y iB and Y iA cells (Fig. 3*A*) and 34–48 h in B iA and A iB cells (Fig. 3*B*). For the studies described here, a 24-h induction time was used. These time course and ponA concentration data are similar to those reported for induction of β-galactosidase in CV-1 cells (26).

Progesterone-dependent Down-regulation of PRs Despite Continuous ponA—Wild-type PRs undergo ligand-dependent down-regulation coincident with strong transcriptional activation (27). To determine whether the inducible PRs exhibit this physiologically important response, Y iB cells were treated with or without ponA for 24 h and then with or without the progestin R5020 for 72 h while ponA was continued (Fig. 4). In the absence of R5020, PR-B were detectable for at least 72 h (Fig. 4*A*, *left panel*). In the presence of R5020, PR-B was ~70% down-regulated by 12 h and 95% down-regulated by 24 h (Fig. 4*A*, *right panel*). This time course of down-regulation was identical to that observed for PR expressed by its endogenous promoters in T47D cells and for PR expression driven by the exogenous SV40 promoter in the T47D-YA and T47D-YB cells. It occurred despite the continuous presence of ponA and indicates that down-regulation is a post-transcriptional phenomenon. The molecular mass upshift of R5020-occupied PR-B in the *right panel* is indicative of ligand-dependent phosphorylation (28–30).

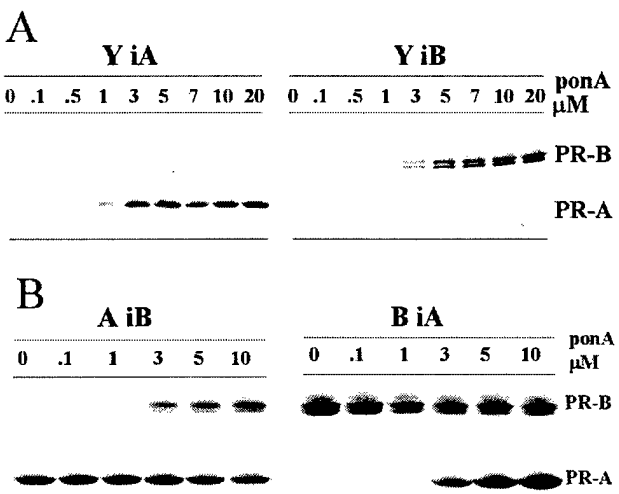

FIG. 2. **A ponA dose of 10 μM is optimal for induction of PR.** Y iA and Y iB (*A*) or A iB and B iA (*B*) cells were treated with vehicle or the specified amounts of ponA for 24 h; cells were harvested; WCEs were prepared; and immunoblotting was performed as described under "Experimental Procedures."

Receptor Turnover in the Absence of ponA—To define the time course of PR disappearance after ponA withdrawal, Y iB cells were induced with ponA for 24 h. ponA was then washed out; cells were treated with or without R5020; and PR-B levels were measured for 0–72 h thereafter (Fig. 4*B*). In the absence of R5020, PR-B declined by 39% at 12 h and by 93% at 24 h following ponA removal (Fig. 4*B*, *left panel*). The PR loss in the absence of R5020 was due to a halt in transcription coupled with protein turnover. Ligand-dependent down-regulation due to R5020 treatment (Fig. 4*B*, *right panel*) accelerated this process, with 98% loss of PR-B by 12 h.

PR-A/PR-B Heterodimers in Transient Transcription Assays—To test whether the induced receptors are functional as homo- or heterodimers, cells were transiently transfected with MMTV (24) or PRE_2-$TATA_{tk}$ promoter-luciferase reporters and treated for 24 h with 1) vehicle, 2) R5020, 3) ponA, or 4) ponA and R5020 (Fig. 5). In Y iA cells, no transcription above basal levels was observed except in set 4, which received ponA and R5020. Transcription induced by PR-A homodimers was lower from the simple PRE_2-$TATA_{tk}$ promoter (2–3-fold) than from the complex MMTV promoter (10-fold) (Fig. 5, *upper left panel*). The pattern in Y iB cells was similar, except for the typically much higher levels of transcription observed with PR-B homodimers from both PRE_2-$TATA_{tk}$ (50-fold) and MMTV (60-fold) due to AF3 of PR-B (Fig. 5, *upper right panel*, *set 4*). Note the 10-fold difference in the scales of the two panels. No stimulation was observed in Y iNull cells transfected with PRE_2-$TATA_{tk}$-Luc and treated with ponA and progesterone, demonstrating that VgRXR is not activated by progesterone and is not functional on a PRE (data not shown).

To study the influence of heterodimers, A iB and B iA cells

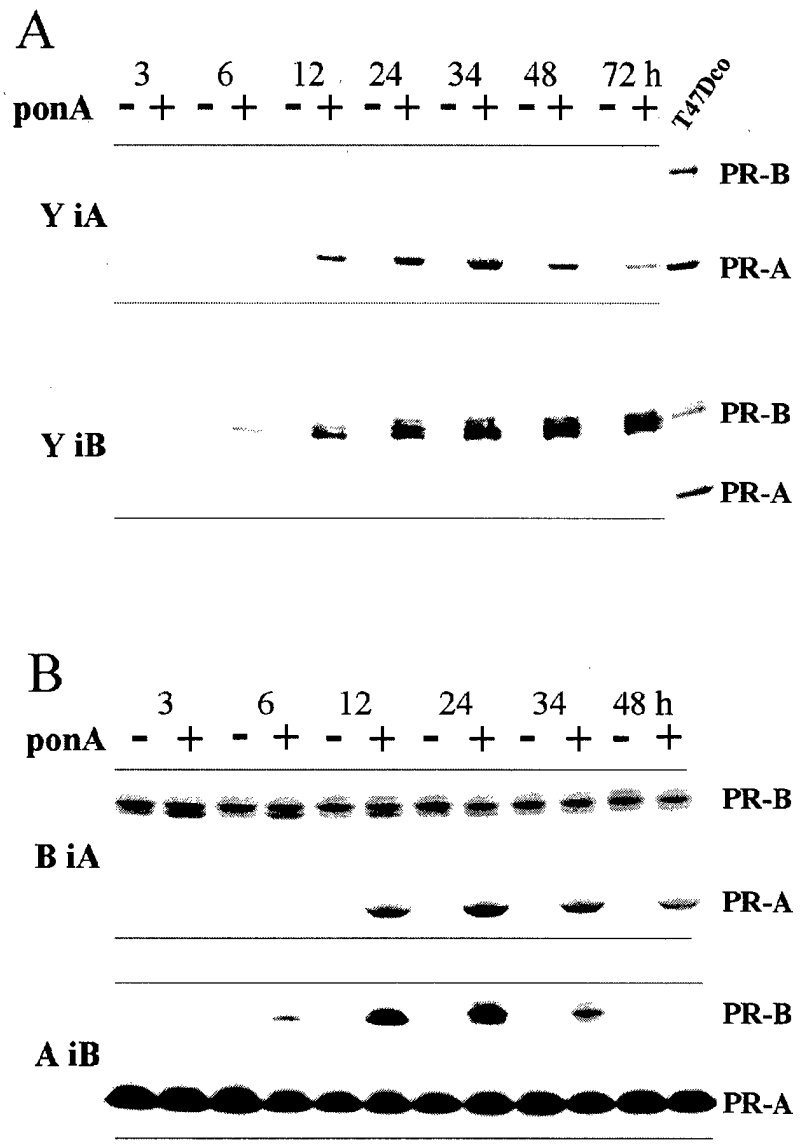

FIG. 3. **Induction of PR is maximal after 24 h of treatment with ponA.** Y iA and Y iB (*A*) or A iB and B iA (*B*) cells were treated with vehicle or 10 μM ponA for the specified times and harvested, and immunoblotting was performed as described under "Experimental Procedures."

were treated with or without ponA for 24 h, followed by vehicle or R5020 for 24 h (Fig. 5, *lower panels*). A iB cells express PR-A constitutively, so in the absence of ponA, R5020 induced the PRE_2 reporter by 3-fold and the MMTV reporter by 20-fold (set 2), as expected for PR-A homodimers. The contribution of PR-B resulting from ponA induction led to marked rises in transcription levels to 15- and 80-fold, respectively (set 4). However, maximum levels in A iB cells (~45,000 luciferase units) did not approach the levels seen in Y iB cells (~200,000 units). At equimolar levels of PR expression, binomial distribution analysis predicts that ~50% of PRs are heterodimers and that 25% are PR-A or PR-B homodimers (31). The repressor contribution of PR-A as the homodimer and/or heterodimer requires further study. Similarly, in the B iA cells, the strong transcription observed with constitutively expressed PR-B homodimers (120–130-fold induction over base-line levels from both reporters) (set 2) was reduced by ~75% (PRE_2-TATA) and 50% (MMTV) upon induction of PR-A (set 4). Thus, paradoxically, although the induced cells contain higher total PR levels, their transcription levels are lower. These experiments document the inhibitory actions of PR-A on PR-B-mediated transcription in transient transfection assays (1, 3), using models in which the mechanisms can be addressed.

AQ: V

Model Cells to Study PR-A Versus PR-B Regulation of Endogenous Genes—In exogenous expression systems, coexpression of the two PRs leads to transcription levels that differs from those seen with each receptor alone (Fig. 5). This has led to the long-held generalization that PR-A are repressors of PR-B activity. Without appropriate experimental models, however, this has not been analyzable on endogenous genes. The new cell lines were designed to address this deficiency since our long-term goal is to analyze this issue in a global manner. In Fig. 6, we show preliminary results using two endogenous genes: *bcl-x_L*, which we previously showed to be up-regulated specifically by PR-A; and tissue factor, which we previously showed to be up-regulated specifically by PR-B (5). We now investigated what happens to the endogenous regulation of each gene when the other PR isoform is added, using the four PR-expressing cell lines. Each transcript was measured by RT-PCR after treatment with 1) vehicle, 2) progesterone, 3) ponA, or 4) ponA plus progesterone. Immunoblotting was performed to quantify PR induction (data not shown, but see Fig. 3).

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Fig. 6*A* shows the data for *bcl-x_L*. The transcript was induced

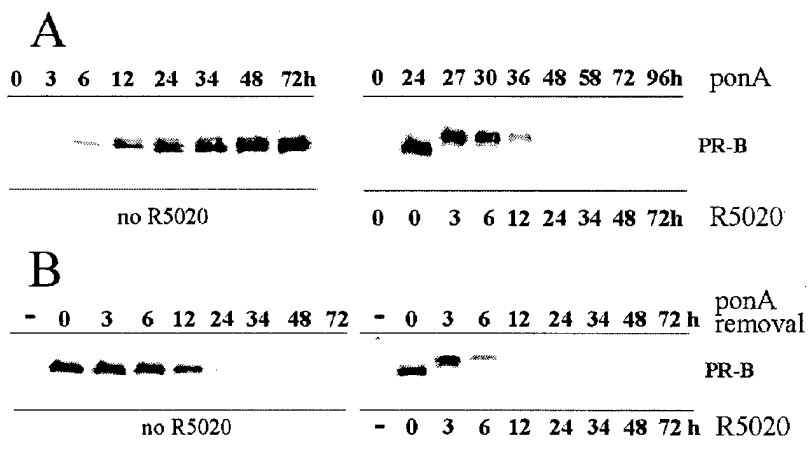

FIG. 4. **Induced PRs undergo appropriate ligand-dependent phosphorylation and down-regulation despite continuous ponA treatment.** *A*, Y iB cells were induced for the specified times with 10 μM ponA without R5020 (*left panel*); or cells were pretreated with 10 μM ponA for 24 h, and R5020 was added with continuous ponA treatment for the specified times (*right panel*). Cells were harvested; whole cell extracts were prepared; and immunoblotting was performed as described under "Experimental Procedures." *B*, Y iB cells were treated with 10 μM ponA for 24 h; then ponA was removed, and no R5020 was added (*left panel*), or ponA was removed and R5020 was added (*right panel*). Cells were harvested at the specified times, and immunoblotting was performed as described under "Experimental Procedures."

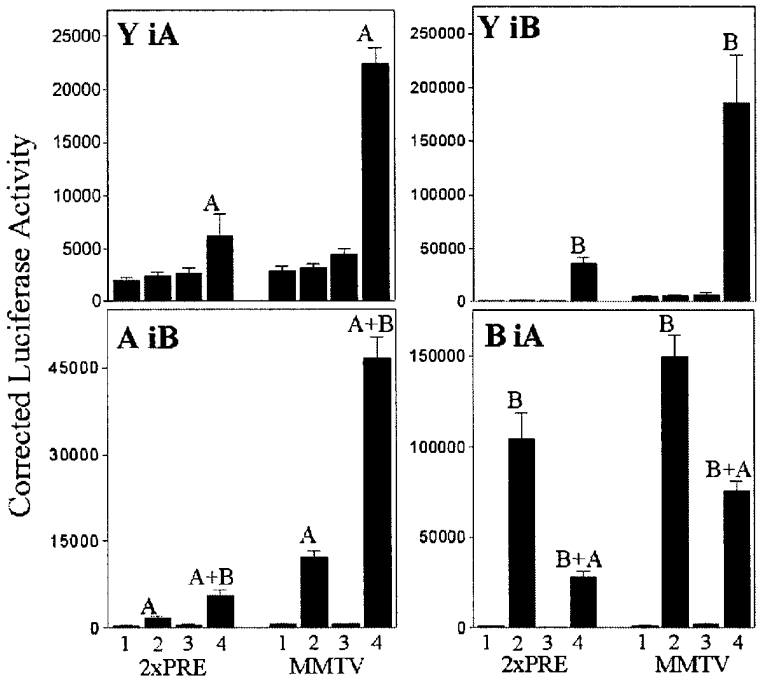

FIG. 5. **Induced PRs are transcriptionally active on exogenous promoters: PR-A suppresses the effects of PR-B.** Y iA, Y iB, A iB, or B iA cells were transiently transfected with PRE_2-Luc or MMTV-Luc. Y iA and Y iB cells were treated concomitantly with Me_2SO + EtOH (*set 1*), Me_2SO + 10 nM R5020 (*set 2*), 10 μM ponA + EtOH (*set 3*), or ponA + R5020 (*set 4*) for 24 h. A iB and B iA cells were induced with vehicle or 10 μM ponA for 24 h, and then vehicle or R5020 was added for an additional 24 h (treatments were the same as listed above). Cells were harvested, and assays were performed as described under "Experimental Procedures." *Bars* indicate S.E. for at least three independent experiments.

2-fold above basal levels in Y iA cells (which express PR-A and were treated with progesterone) only in set 4. In A iB cells, the transcript was again weakly up-regulated by PR-A (1.8-fold; set 2). Expression of approximately equimolar PR-B increased transcription (2.4-fold; set 4). This suggests the interesting possibility that the PR-A/PR-B heterodimer may also regulate *bcl-x_L* transcription. Furthermore, *bcl-x_L* was also up-regulated by progesterone in T47Dco cells, which coexpress PR-A and PR-B (5). In B iA cells, *bcl-x_L* transcription was not regulated by PR-B (set 2) and was up-regulated when PR-A was induced and progesterone was added (set 4).

What about the tissue factor, which is specifically regulated by PR-B? Fig. 6*B* shows 4.5-fold up-regulation of tissue factor in Y iB cells (set 4), which express PR-B and were progesterone-treated for 6 h. In A iB cells, tissue factor was poorly (1.4-fold) regulated by PR-A (set 2); and surprisingly, addition of PR-B had little effect (1.6-fold; set 4). Is constitutive PR-A repressive? Two time points are shown using B iA cells. In the first, at 6 h of progesterone treatment, tissue factor was up-regulated by constitutive PR-B (4.6-fold; set 2). Induction of PR-A to an ~2-fold molar excess over constitutive PR-B and addition of progesterone (set 4) decreased tissue factor levels somewhat (compare set 4 with set 2). This weak inhibitory effect of induced PR-A was entirely absent at 12 h of progesterone treatment. This study illustrates the complexity of the issues we are trying to address because it raises the further possibility that on some genes, the pre-existing receptor is dominant over the fluctuating one and that effects of PR isoforms on gene regulation vary over time. This would have implications in tissues like the uterus, in which the PR-A/PR-B ratio varies extensively during the menstrual cycle due to fluctuations in the levels of PR-B (7, 8). AQ: W

Model Cells to Study Ligand-independent Effects of PRs—These new cells are also uniquely suited to study ligand-independent gene regulation by PRs. This is demonstrated in Fig. 7 by microarray methods using Y iA cells; Y iNull cells served as controls for VgRXR activity. Cells were treated with 1) vehicle, 2) progesterone (6 h), 3) ponA, or 4) progesterone (6 h) plus ponA. RNA was extracted, and poly(A)$^+$ RNA was prepared, derivatized, and hybridized to Affymetrix chips (5) displaying ~12,000 human genes. Data for three (Y iA) or two (Y iNull) time-separated experiments were generated and analyzed sta- F7

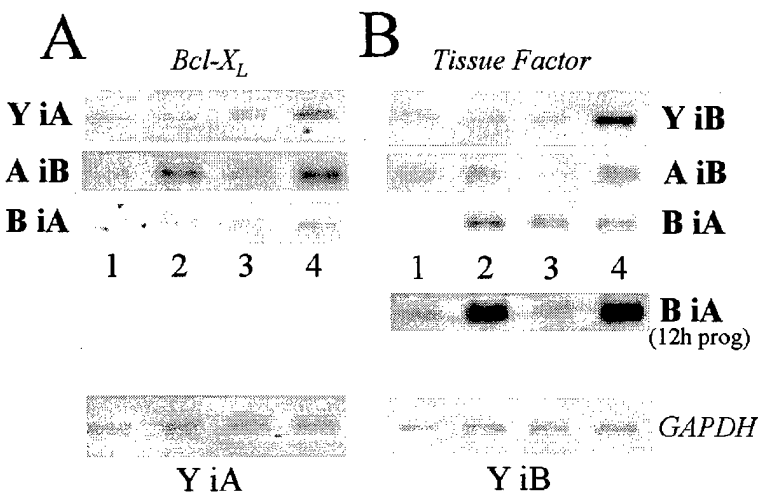

FIG. 6. **Induced PRs regulate endogenous progesterone-responsive genes.** Y iA, A iB, and B iA cells (*A*) or Y iB, A iB, and B iA cells (*B*) were treated with Me_2SO (*lanes 1* and *2*) or 10 μM ponA (*lanes 3* and *4*) for 24 h, and then EtOH (*lanes 1* and *3*) or 10 nM progesterone (*prog*; *lanes 2* and *4*) was added for 6 or 12 h for B iA cells where indicated. Cells were harvested; total RNA was prepared; and RT-PCR was performed with *bcl-x_L*, tissue factor, or GAPDH primers as described under "Experimental Procedures." Samples were resolved on 2% agarose gels and visualized by reverse imaging of ethidium bromide-stained gels. Densitometry was performed using an Alpha Imager.

tistically (5). Results for four genes are described (Fig. 7); *asterisks* denote statistically significant ($p < 0.01$) induction by unliganded PR-A (set 3) over PR-negative states (sets 1 and 2) in Y iA cells. None of the genes were regulated in Y iNull cells. The four genes shown are the cell cycle inhibitor p21 (Fig. 7*A*), *ENC1* (ectodermal-neural cortex 1) Fig. 7*B*), the cell adhesion molecule (*PCDH1*) (Fig. 7*C*), and PRLR (Fig. 7*D*). The effect of unliganded PR-A on the PRLR gene was subtle (1.7-fold), but reproducible and statistically significant ($p < 0.01$). Because PRLRs are of interest in the breast, we confirmed the results by RT-PCR (Fig. 7*E*). PRLR transcript levels increased 1.8-fold after ponA induction of PR-A (set 4) in three independent experiments. The PR requirement was confirmed by the ability of RU486 to suppress the ponA component (set 5) without affecting basal levels (set 2).

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DISCUSSION

The Models—The ecdysone-inducible system produces tight, ponA dose-dependent regulation of PRs. These models allow us to isolate the effects of each PR isoform and to vary isoform ratios while controlling for confounding factors such as differences in the genetic backgrounds of cells. The receptors retain wild-type biochemical properties as monitored by their ligand-dependent down-regulation, ability to be phosphorylated, and transcriptional regulation of endogenous genes and exogenous promoters. Control cells that contain VgRXR and an inducible construct lacking the PR cDNA insert (Y iNull) assess pleiotropic effects, if any, of the VgRXR regulatory heterodimer. With regard to other control issues, wild-type ecdysone receptors utilize the same chaperone and some co-regulator proteins as steroid receptors (32, 33), and this could theoretically impact PR expression and/or function. However, PRs are produced at high levels in these cells, even when VgRXR activation is prolonged by ponA treatment (Fig. 3). Thus, it is unlikely that chaperones are limiting. Similarly, although it is unknown whether the modified ecdysone receptors and PRs utilize common co-regulatory proteins, the very high transcription levels obtained with progestins and the excellent repression obtained with the antiprogestin RU486 (data not shown) suggest that co-regulators are also not limiting. In addition to controlling expression of each isoform independently, two of the cell lines allowed us to overexpress one isoform over the other by ~2-fold (Fig. 2*B*). We anticipate generating even larger excursions in this ratio by adding a rexinoid to the ponA (26). Other uses of these cells are to study the PR dependence of non-genomic effects of progesterone (34, 35) and to study ligand-independent effects (see below).

AQ: Z

Ligand-independent Effects of PRs—The Y iA cells demonstrated ligand-independent regulation by human PR-A of a subset of endogenous genes, four of which are shown in Fig. 7 for illustrative purposes. In preliminary experiments, we found a much larger number of genes uniquely regulated by unliganded PR-A than by unliganded PR-B. This is surprising because the opposite is the case for regulation by ligand (5). Ligand-independent effects of nuclear receptors are unusual, and the mechanisms are unclear. Chicken PR-A is activated in a ligand-independent manner by cAMP and epidermal growth factor (36, 37). Despite attempts to do so, this phenomenon has not been reliably demonstrated for human PRs (38). In addition to chicken PRs, several other nuclear receptors can be activated by dopamine through D1 receptors (39), and dopamine placed directly into the third ventricle of the brain increases female rat sexual behavior in a progesterone-independent, but PR-dependent manner (40). Interestingly, two genes regulated by PR-A in a ligand-independent manner, *ENC1* and the cell adhesion molecule *DSCAM* (data not shown), are expressed at high levels in neuronal cells and may be additional brain targets of unliganded PRs. We can only speculate about the mechanisms for ligand-independent gene regulation by PRs. Treatment with 8-bromo-cAMP does not alter chicken PR (41) or human PR (42) phosphorylation, suggesting that the direct target for phosphorylation by this signaling pathway may be one or more transcriptional co-regulatory proteins. For example, the co-regulatory protein SRC-1 is phosphorylated following cAMP treatment and increases the transcriptional activity of PRs (43).

PRLRs are known to be progesterone-regulated (44). Their expression is complex and involves multiple tissue specific promoters. The rat PRLR gene contains tissue-specific promoters that are regulated by several transcription factors, including SP1, STAT5, and CAAT/enhancer-binding protein-β (45, 46). Two promoters, PIII and P_N, drive PRLR expression in human cells (47). The human P_N promoter contains a putative nuclear receptor-binding site, but neither promoter contains a consensus PRE. However, there are putative SP1-binding sites in both PRLR promoters (47). These SP1 sites are of interest because we have previously shown that progesterone regulation of the promoter for the cell cycle inhibitor p21 is indirect, through tethering of PRs to SP1 (48) rather than binding to PREs. These studies also demonstrated that unliganded PR-A, but not PR-B, interacts directly with SP1 (48), perhaps explaining the greater ligand-independent transcriptional efficacy of PR-A that we observed in the present study (Fig. 6*B*). Note that p21 is also regulated by PR-A in a ligand-independent manner (Fig. 7*A*). The physiological relevance of ligand-independent

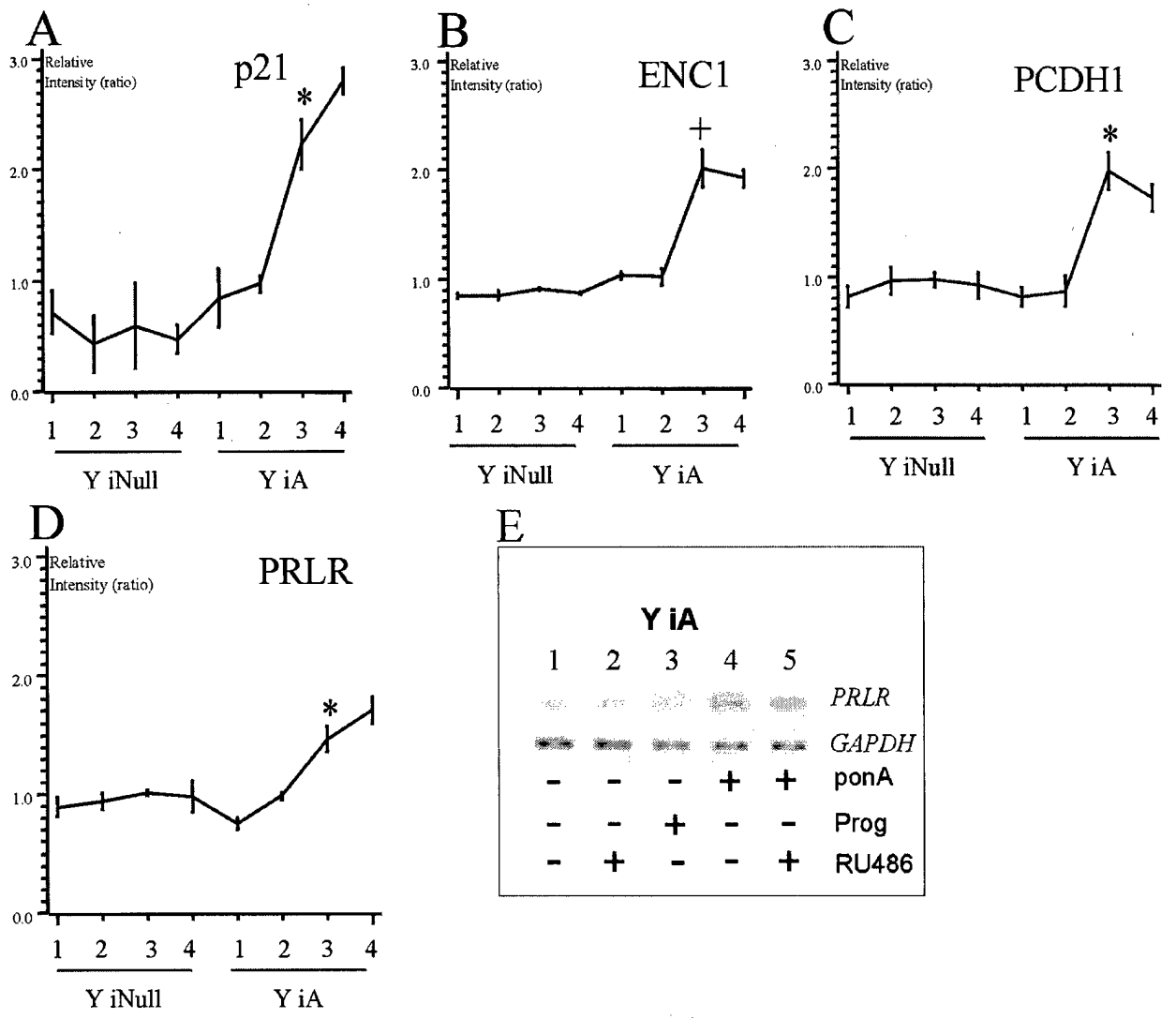

FIG. 7. **PR-A regulates genes in a ligand-independent manner.** Y iA (three independent experiments) or Y iNull (two independent experiments) cells were induced in MEM with CSS containing Me_2SO or 10 μM ponA for 24 h, and then EtOH or progesterone was added for 6 h. The four treatment groups were as follows: *set 1*, Me_2SO + EtOH; *set 2*, Me_2SO + progesterone; *set 3*, ponA + EtOH; *set 4*, ponA + progesterone. Poly(A)$^+$ RNA was prepared and derivatized; microarray analysis was performed; and data were analyzed as previously described (5). *A*, p21 gene; *B*, *ENC1*; *C*, *PCDH1*; *D*, PRLR gene. *Bars* represent the range of values for duplicate experiments (Y iNull cells) and S.E. of triplicate experiments (Y iA cells). The symbols denote statistically significant differences (*, $p < 0.01$; +, $p < 0.001$) as assessed by one-way analysis of variance, followed by a Tukey multiple comparison test between the PR-negative set (set 1) and the parallel ponA-treated set (set 3). The difference between the two progesterone-treated sets (PR-negative (*set 2*) *versus* PR-positive (*set 4*)) is also statistically significant. *E*, Y iA cells were treated with Me_2SO or 10 μM ponA for 24 h, and then 100 nM RU486 or 10 nM progesterone (*Prog*) was added for 6 h where indicated. Cells were harvested; total RNA was prepared; and RT-PCR was performed with primers specific for PRLR or for GAPDH as a control.

gene regulation is unknown. Because PRLRs and PRs are coexpressed in immature mouse mammary epithelial cells (49) and both genes are expressed in normal breasts of postmenopausal women (50, 51), it is conceivable that ligand-independent mechanisms are engaged during such progesterone-deficient states.

PR-A/PR-B Ratios—At the present time, total PR levels are routinely measured in breast cancers as a guide to therapy. However, given the important functional differences between PR-A and PR-B, summing the levels of the two receptors to arrive at this total is uninformative. In fact, we show here, using exogenous promoters, that as the contribution of PR-A to total PR levels increases, transcription levels can paradoxically decrease (Fig. 5). However, regulation of endogenous genes may be more complex, depending, among other things, on the maturity of the receptors and the treatment time. Because PRs are post-translationally modified by sumoylation and by phosphorylation in a time-dependent manner after protein synthesis (52), this could provide an explanation for differences in function between nascent and mature receptors. These new cell lines provide ideal models to study post-translational modifications and their effects on receptor maturation and biological activity.

Our studies suggest that the dominant-negative effect of PR-A on PR-B may be promoter-specific and may differ on exogenous promoters *versus* endogenous genes. Interestingly, in progesterone-treated mammary carcinoma cells, the stably integrated MMTV promoter is activated by constitutively expressed PRs, whereas transiently expressed PRs fail to activate transcription (53). Endogenous progesterone-responsive genes may also be differentially regulated depending on whether PR expression is transient or constitutive. Our inducible cells provide a unique model system to examine this question, as PR-A or PR-B can be constitutively or transiently expressed depend-

AQ: AA

ing on whether ponA treatment is continuous or temporary. We are also in a position to study how the ratio of the two isoforms influences transcription of endogenous genes in human breast cancer cells. Several studies indicate that an imbalance in the PR-A/PR-B ratio is physiologically damaging. Breast cancers with an excess of PR-A are less differentiated than tumors with balanced levels of the two isoforms (15). PR-A and PR-B are both present in the normal endometrium. However, only PR-A is detectable in endometriosis (54), whereas overexpression of PR-B is associated with highly malignant forms of endometrial, cervical, and ovarian cancers (55, 56). Equimolar levels of the two PR isoforms have been detected in normal human brain cells, but human chordomas express an excess of PR-B, which is associated with abnormal cell growth (57). Thus, an imbalance in the PR-A/PR-B ratio appears to alter cell growth and other cellular responses to progesterone, but little is known about the underlying mechanisms. The cell lines described here will allow us to investigate these mechanisms.

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AQ: EE

Appendix C

each of thousands of sites within living cells. C/EBPα formed d[illegible]ffectively at most locations throughout the nucleus. Tagging different domains [illegible], and measuring the amount of inter-domain FRET, demonstrated that the [illegible]n of C/EBPα was different at the peri-centromeric chromatin than in the [illegible] nucleus. Treating the cells with an activator of protein kinase C changed [illegible]re of C/EBPα. For some nuclear receptors, we found that dimers were dist[illegible]enly throughout the nucleus. Cognate ligands increased (estrogen receptor [illegible]o and heterodimers), decreased (thyroid hormone receptor homodimer) or [illegible]ffect on (RXRα homodimer, TRβ/RXRα heterodimer) the number of sub[illegible] at which interactions occurred. Some ligands also changed the extent of int[illegible]ach site. For the ER, we found differences in the types of dimerizations ar[illegible]ns promoted by different ligands used in breast cancer therapy. Thus, the [illegible] of conformations of gene regulatory complexes are determined by intranuclea[illegible]. The re-location of co-regulatory factors by some transcription factors may regula[illegible]he patterns of gene expression in differentiation.

OR41-3

New Transcriptional Coregulators of Progesterone Receptors Discovered by Microarray Methods.

Jennifer K Richer*[1], Nicole G Manning[1], Kathryn B Horwitz[1]. [1]*Med, Div of Endocrinology, Univ of Colorado Hlth Scis Ctr, Denver, CO.*

We have found, using microarray analysis of T47D human breast cancer cells, that members of the Kruppel-like zinc finger, homeobox, and winged helix domain families of transcription factors are upregulated by 6 hrs of progesterone treatment. These transcription factors were not previously known to be progesterone, or progesterone receptor (PR) dependent. PR regulation has been confirmed by RT-PCR, northern, or western blot analysis. Progesterone directly upregulates expression of these mRNAs at early time points (1.5 and 3.0 hrs) in a cycloheximide insensitive manner. To test the theory that these immediate early transcription factors, in turn affect PR activity, we examined their effects on transcription of PRE2tata, MMTV, and other progesterone-responsive gene promoters linked to a luciferase reporter. The Kruppel-like factor, KLF4, reported to be overexpressed in breast cancers, is upregulated 6 to 8-fold by progesterone. While it upregulates basal transcription, it inhibits PR-dependent transcriptional activity by 85% in a dose-dependent manner. The winged helix protein, FKHR, is also upregulated by progesterone treatment. Transient expression of FKHR increases PR-dependent transcriptional activity 8 to 10-fold without altering basal transcription. Both KLF4 and FKHR interact with PR as shown by immuno-precipitation assays. The mechanisms of action of KLF4 and FKHR suggest that both can be classified as new nuclear receptor coregulatory proteins. Our data establish the interesting principle that factors regulated by progesterone, can in turn regulate progesterone's activity, and emphasize novel applications of microarray data.

Supported by the National Institutes of Health (DK48238 and CA26869), National Foundation for Cancer Research (10COL3), and Department of Defense Breast Cancer Program (BC996535).

OR41-4 ♦ ⊗

Bioinformatics Tools for Functional Genomi[illegible] Endocrinology.

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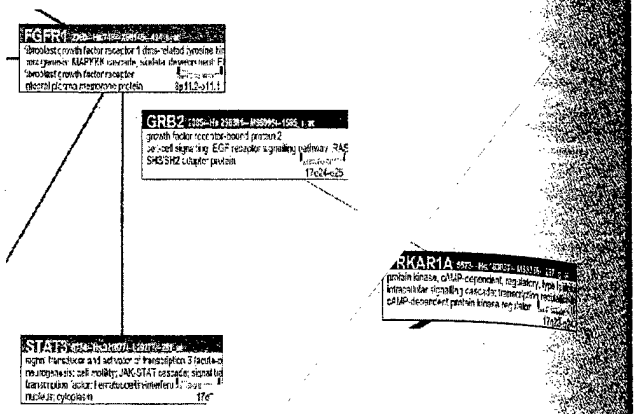
With current microarrays, a single exp[illegible]w provide quantitative information on the expression of 60,000 [illegible]though this can be used to determine the transcriptional response [illegible]ti, novel tools are still needed to sift through huge databases of RNA [illegible]ind true nuggets of related function. Here we describe the applicati[illegible]ges we have made freely available to the functional genomics comm[illegible]termed **relevance networks**, comprehensively compares all measured [illegible]otypic measurements against each other, and builds networks of gener[illegible]s from the highest scoring pairs. Advantages of this method includ[illegible]ssociations are shown (e.g. those from tumor suppressing genes), (2[illegible] types can be included (i.e. clinical and expression), and (3) multip[illegible] are allowed (e.g. a transcription factor may regulate the expression of [illegible]nan a helicase). In the figure, we show relevance networks linking end[illegible]ated FGFR1 with STAT3 and GRB2 with PRKAR1A, from an analysis of medul[illegible]oblastoma samples.



We have also dev[illegible]database and freely available web-site call[illegible] **www.unchip.org** that ta[illegible]on codes and returns the latest information about each microarray probe set[illegible]g official names, molecular functions, chromosomal positions, and prote[illegible]. For the first time, this allows one to return to hypothesis based analysis o[illegible]ays; for instance, one can analyze microarrays with prior knowledge, sur[illegible]a of chromosome 4 or proteins containing SH2 domains being involved.

Hav[illegible]d over 600 microarrays with these and other tools, we have example for and have[illegible]d (1) not all pathways will be reverse engineered using microarrays, (2) looking for simultaneous gene associations ignores the fact that biology takes time, (3) a discovered diagnostic model does not equal the underlying molecular physiology, (4) with rapidly changing information about genes already measured, one is never truly finished analyzing a dataset, and (5) endocrine-related genes and associations are in all tissues.

OR41-5 ♦

A Genomic Approach To Identify Novel Progesterone Receptor-Regulated Pathways in the Uterus during Implantation.

Yong-Pil Cheon*[1], Quanxi Li[1], Francesco J DeMayo[2], Indrani C Bagchi[1], Milan K Bagchi[3]. [1]*Veterinary Biosciences, Univ of Illinois at Urbana-Champaign, Urbana, IL; [2]Molecular and Cell Biology, Baylor Coll of Med, Houston, TX; [3]Molecul Integrative Physiology, Univ of Illinois at Urbana-Champaign, Urban*

The steroid hormone progesterone (P) plays a profound role [illegible]ng cell growth and differentiation programs in the reproductive tract du[illegible]ancy. The cellular actions of P are mediated via its nuclear receptors, which [illegible]e expression of specific target genes. The identities of gene networks reg[illegible]ne P receptors (PRs) at various stages of pregnancy, however, remain largel[illegible]. In this study, we have utilized oligonucleotide microarrays to identify mP[illegible]e expression in the pregnant mouse uterus is modulated by RU486, a PR an[illegible]ich is also an effective inhibitor of implantation. We found that in respons[illegible], expression of mRNAs corresponding to seventy-nine known genes was d[illegible]ted two-fold or more in the preimplantation mouse uterus. These genes i[illegible]veral previously reported P responsive genes as well as many novel ta[illegible]. The PR regulation of several microarray-derived genes was ascertained[illegible]istering P to ovariectomized wild type and PR knockout (PRKO) mice. W[illegible]at P induced the expression of these genes in the uteri of wild type but not P[illegible]. The novel PR-regulated genes included p12 (serine protease inhibitor), cy[illegible]ymphocyte antigen-2b (cystein protease inhibitor), fisp 12 (growth factor[illegible]- and epidermal-12/15-lipoxygenases (lipid metabolizing enzymes), folli[illegible]ptide hormone), osteoblast-specific factor-1 (transcription factor), immur[illegible]ve gene I (cell adhesion molecule), and calcyclin (calcium binding protein)[illegible]entified seventy known genes that were up regulated two-fold or more in th[illegible] uterus in response to RU486. Interestingly, several RU486-inducible ge[illegible]ansferrin, Muc-1, carbonic anhydrase II and epithelial zinc finger protein[illegible]nduced by estrogen (E) in the uteri of ovariectomized mice. This finding sur[illegible]view that PR represses the expression of many E-regulated genes in the P-de[illegible]a pregnant uterus, and binding of RU486 to the receptor reverses this effect. The ide[illegible]fication of several novel PR-regulated gene pathways in the reproductive tract is an important step towards understanding how P regulates the physiological events leading to implantation.

253 **New Transcriptional Coregulators of Progesterone Receptors Discovered by cDNA Microarray Methods**

Jennifer K. Richer, Nicole G. Manning, and Kathryn B. Horwitz
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By examining progesterone regulated genes in progesterone receptor (PR) positive T47D breast cancer cells using microarray analysis, we have found that members of the Kruppel-like zinc finger, homeobox, and winged helix domain family of transcription factors are upregulated by PR after 6 hrs of hormone treatment. These transcription factors were not previously known to be progesterone regulated. We have confirmed the regulation of these factors by PR, using RT-PCR, northern or western blot analysis. PR directly regulates these transcription factors in the presence of cycloheximide at early time points (1.5 and 3.0 hrs) of progesterone treatment.
Other transcripts encoding proteins already known to bind to PR and affect their activity were also found to be regulated by PR in our study. Therefore, to test the theory, that the immediate early transcription factors which we have found to be PR regulated might in turn affect PR activity, we examined their effects on transcription of PRE_2tata, MMTV, and other progesterona-responsive gene promoters linked to a luciferase reporter. One of the Kruppel-like factors strongly upregulates basal transcription but inhibits transcriptional activity of progesterone occupied PR on PRE_2tata-luc in a dose-dependent manner by 85%. The winged helix domain protein, on the other hand, increases PR-dependent transcriptional activity 8-10-fold, without altering basal transcriptional activity.
These data establish the interesting principle, that factors which are regulated by progesterone, can in turn regulate progesterone's activity. They underscore an important use of array data. We are analyzing the role of these new coregulatory proteins in the normal and malignant breast. Supported by the National Institutes of Health (DK48238 and CA26869), National Foundation for Cancer Research (10COL3), and Department of Defense Breast Cancer Program (BC996535).

254 **Activation and Repression: Factor Recruitment and TIF2/GRIP1 Corepressor Activity at a Collagenase-3 AP-1 Element/Tethering GRE**

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Glucocorticoid receptor (GR) represses transcription mediated by AP-1 and NF-kB, which drive the expression of pro-inflammatory cytokines, cell adhesion molecules and tissue-degrading enzymes, such as collagenases. We examined factor occupancy and function at an AP-1 response element, col3A [illegible]iated with the collagenase-3 gene in human U2OS osteosarcoma cells; col3 [illegible]fers activation by phorbol esters, and repression by glucocorticoid and thyr[illegible]mones. The subunit composition and activity of AP-1, which binds col3A, paralleled the intracellular level of cFos, which is modulated by phorbol esters [illegible]ucocorticoids. In contrast, a similar AP-1 site at the collagenase-1 gene, no[illegible]cible in U2OS cells, was not bound by AP-1. GR associated with col3A th[illegible] protein-protein interactions with AP-1, regardless of AP-1 subunit compos[illegible] and repressed transcription. TIF2/GRIP1, reportedly a coactivator for GR[illegible]ne thyroid hormone receptor (TR), was recruited to col3A and potentiated [illegible]ediated repression in the presence of a GR agonist but not an antagonist. In [illegible]onal assays monitoring AP-1 reporter activity, GRIP1 mutants deficient in GR[illegible]ing and coactivator functions were also defective for corepression, and a C[illegible] fragment containing the GR-interacting region functioned as a domin[illegible]egative for repression. Similarly, IL-8 gene-derived reporters, containin[illegible] AP-1 and NF-kB elements, or NF-kB element alone, were also co-repressed[illegible]ull length GRIP1 and de-repressed by dominant-negative GRIP1. In cor[illegible] TIF2/GRIP1 was not recruited to col3A as part of TR repression complex an[illegible] not potentiate TR-mediated repression of AP-1 in functional assays. Interesti[illegible] our mapping and sequence analysis suggests that this corepressor functic[illegible]TIF2/GRIP1-specific and does not extend to other p160 family members. Thus, the composition of regulatory complexes, and the biological activities of the bound factors, are dynamic and dependent on cell and response element contexts. Cofactors such as GRIP1 likely contain distinct surfaces for activation and repression that function in a context-dependent manner.
(NIH-CA-20535 and the Parker B. Francis Postdoctoral Fellowship in Pulmonary Research from the Francis Families Foundation).

255 **Role of PR-A vs. PR-B in Estrogen-Dependent Human Breast Tumor Growth**

Carol A. Sartorius, Tianjie Shen, and Kathryn B. Ho[illegible]
Division of Endocrinology, University of Color[illegible]ealth Sciences Center, Denver, CO, USA, 80262.

Approximately two thirds of ER positive[illegible]an breast tumors that respond to endocrine therapy are also [illegible]positive. However, PR positive tumors contain varying rat[illegible] of the two naturally-occurring receptor isoforms, PR-A and PR-[illegible]R-A and PR-B have different functions *in vivo* including diff[illegible]dial effects on estrogen mediated actions in target tissues. To [illegible]y how each PR isoform influences tumor growth, we have d[illegible]oped a model system in which human T47D breast cancer cel[illegible]xpressing either PR-A or PR-B are grown into solid tumors in [illegible]ectomized female nude mice. Growth of tumors *in vivo* oc[illegible]in a strictly estradiol-dependent manner. Our data show that [illegible]status, independent of ligand, affects estradiol-dependent t[illegible]r growth. In particular, cells expressing PR-B grow significar[illegible]arger tumors, on average, than cells expressing PR-A. This o[illegible]s with independently derived PR expressing cell lines and des[illegible]he fact that PR-A and PR-B containing cells have app[illegible]ximately equal population doubling times in culture. Addition of *both* estrogen and progesterone at initial tumor formation did not alter growth patterns. Differences in growth of established tumors were observed, however, between PR-A and PR-B containing tumors treated with either tamoxifen or the antiprogestin RU486. These experiments will help determine which types of tumors may be the best candidates for hormone therapies. *This work was supported by DAMD BC996108 and the Cancer League of Colorado.*

256 **ERAP140, a conserved tissue-specific estrogen receptor coactivator**

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Estrogen plays a central role in the control of development, sexual behav[illegible]d reproductive functions, and its actions modulate the progression of a varie[illegible]uman diseases, including breast and endometrial cancers, cardiovascula[illegible]ase and osteoporosis. The diverse biological effects of estrogens are medi[illegible]rimarily by estrogen receptors alpha and beta (ERα, ERβ). However, it has b[illegible]e clear that the expression of the full spectrum of responses to estrogen r[illegible]s the action of a number of ER coregulatory factors that modulate targe[illegible] transcription in an orchestrated fashion.

Using the ERα ligand-binding domain [illegible]) as a probe to screen for interacting proteins, we have identified a novel r[illegible]n, ERAP140, that binds ERα in an agonist-, but not antagonist-dependent [illegible]er. The ERAP140 protein shares no sequence and little structural homolog[illegible] other nuclear receptor cofactors. However, homologues of ERAP140 hav[illegible]n identified in mouse, *Drosophila* and *C. elegans*. The expression of ERAP[illegible] cell- and tissue-type specific, being most abundant in the brain where its e[illegible]ion is restricted to neurons. In addition to interacting with ERα, ERAP140[illegible]s other nuclear receptors including ERβ, TRβ, PPARγ and RARα. We hav[illegible]pped the interaction of ERAP140 with ERα to a non-canonical interactio[illegible]f. However, the ERα-ERAP140 association can be competed by p160 co[illegible]ior NR boxes, indicating ERAP140 binds ERα on a surface similar to o[illegible]coactivators. When co-expressed, ERAP140 enhances the transcriptional a[illegible]y of ERα and the other nuclear receptors with which it can interact. More[illegible]rtantly, ERAP140 is recruited *in vivo* by estrogen-bound ERα to the promote[illegible]ion of endogenous ERα target genes in a cyclic pattern consistent with the action of other ER coactivators. Our results demonstrate that ERAP140 represents a distinct class of nuclear receptor coactivators that can modulate receptor signaling in specific target tissues.
This work was supported by NIH grant RO1-CA57374 and by a fellowship from Massachusetts Department of Public Health Breast Cancer Research Program

Appendix D

PII Redacted

JENNIFER RICHER, Ph.D.
CURRICULUM VITAE

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Education:

Ph.D. 1992, Colorado State University, Ft. Collins, CO. Dept. of Pathology, Cell and Molecular Biology Interdisciplinary Program.
M.S. 1988, University of Texas, El Paso, TX. Dept. of Biological Sciences
B.S. 1986, University of Texas, El Paso, TX.
Major: Biology, Minor: Chemistry

***Relevant Employment*:**

4/2000-present	Assistant Professor, Division of Endocrinology, Dept of Medicine UCHSC, Denver, Colorado
9/1996-4/2000	Instructor, Endocrinology Division, UCHSC, Denver, Colorado
9/1994-1996	Postdoctoral Fellow with Kathryn B. Horwitz, Ph.D.Endocrinology Division, UCHSC, Denver, Colorado.
5/1992-9/1994	Postdoctoral Fellow with Claude Maina, Ph.D. New England Biolabs, Beverly, Massachusetts
1988-1992	Research Assistant with Dr. Robert Grieve, Colorado State Univ. Ft. Collins, Colorado
1986-1988	Teaching Assistant, Dept. of Biological Sciences, Univ. of Texas, El Paso
1983-1987	Writing and Science Tutor, Univ. Study Skills and Tutorial Services, Univ. of Texas, El Paso,Texas.

Current Funding:

2002-2005	Molecular Determinants of Response to Endocrine Therapy for Breast Cancer Department of Defense Clinical Bridge Award: **Principal Investigator: Jennifer Richer, Total Costs: $434,329.**
2002-2003	Avon Foundation Seed Grant **Principal Investigator: Jennifer Richer, Total Costs: $50,000.**
2001-2002	Department of Defense Breast Cancer Research Program Concept Award: BC996535 "Role of Progesterone Receptor Isoforms in Regulation of Cell Adhesion and Apoptosis." **Principal Investigator: Jennifer Richer, Total Costs: $75,000.**
2001-2002	University of Colorado Center Cancer/American Cancer Society Center Seed Grant. "Differential Progesterone Receptor Isoform Function Determines Breast Cancer Cell Fate." **Principal Investigator: Jennifer Richer, Total Costs: $20,000**

Scholarships/Fellowships/Patents and Awards:

2000	U.S. Patent Application No. 09/814,916 "Progesterone Receptor-Regulated Gene Expression and Methods Related Thereto" Inventors Jennifer Richer, Kathryn Horwitz.
2000	Travel Grant to Endocrine Society Meeting, Toronto CA -Women in Endocrinology
2000	University of Colorado Project No. 10091H Provisional Patent Application "Surrogate Gene Markers for Two Different Progesterone Receptor Isoforms in Breast Cancers."
1995-1996	Thorkildesen Fellowship for Cancer Research, UCHSC. Principal Investigator: Jennifer Richer Total Costs: $35,000
1993-1995	NIH SBIR Grant, "Identification of Ecdysone Receptor in Nematode Parasites" Co-investigators: Jennifer Richer with Claude Maina at New England Biolabs. Total Costs: $250,000
1995	US Patent Application No 08/482,282 entitled "Novel Dirofilaria and Onchocerca Larval L3 Cystein Protease Proteins and Uses Thereof."
1988-1991	USDA Biotechnology Training Grant-Colorado State Univ.
1990-1991	Graduate Fellowship-Cell and Mol. Bio. Program, Colorado State
1988, 1991	Best Student Presentation-Rocky Mountain Conference of Parasitologists
1987-1988	Graduate Scholarship-Univ. of Texas at El Paso
1987-1988	President, Tri-Beta Biological Honor Society
1982-1986	Member University of Texas Honors Program
1982-1984	Undergraduate Scholarship, Univ. of Texas at El Paso

Membership in Professional Societies and Service*

2002	*Ad hoc* reviewer NIH Biochemical Endocrinology Study Section Feb. 2002
2001	Grant Review Panel for Endocrinology #1 section of the Department of Defense Breast Cancer Research Program
2000-present	University of Colorado Cancer Center, Developmental Therapeutics Program – Full Membership
1998-present	Reviewer for Molecular Endocrinology and Breast Cancer Research and Treatment
1998-present	Endocrine Society – Full Membership
1999-present	*Komen Foundation, Denver Chapter, Newsletter contributor and Pink Tie Affair Committee and Speakers Bureau
1990-1991	Research Seminar Committee, Cell and Molecular Biology Program, CSU
1989-1992	Academic Advisory Committee, Cell and Molecular Biology Program, CSU
2000-present	University of Colorado Cancer Center, Developmental Therapeutics Program – Full Membership

Teaching and Mentoring Experience:

2002 Summer	Training of NIH Medical Student Fellow Carrie Marshall, Jointly received $4,000 for her salary from the Endocrine Society
2000-present	Training of Clinical Instructor Dr. Radhika Ghatge, WRHR NIH Training Grant, Division of Obstectrics and Gynecology, Dept of Medicine, MD Fellows Program
1999-present	Training of post-doctoral fellow Dr. Britta Jacobson
1999,2000	Molecular Biology of Cancer - Med 6626, UCHSC, Lecture on Hormone Dependant Cancers
1995-96	Training of Oncology Fellow, Dr. Ann Weirman

1992	Biology of Disease Agents -Parasitology Lectures, College of Veterinary Medicine and Biomedical Sciences, Colorado State University
1990-91	Agents of Disease Laboratory, College of Veterinary Medicine and Biomedical Sciences, Colorado State University
1986-88	Teaching Assistant for the following laboratories Univ. of Texas: Topics in the Study of Life, Anatomy and Physiology, Transmission Electron Microscopy Techniques, Molecular Cell Biology

Publications:

Richer, J.K , Jacobson, B., Manning, N.G., and Horwitz, K.B. 2002. Functional differences between progesterone receptor isoforms: differential gene regulation in breast cancer cells. J Biol Chem 277: 5209-5218. 2002

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Richer, J.K. and K.B. Horwitz. 8/1999. Differential Roles of Progesterone Receptor Isoforms in Breast Cancer. **Invited Oral Presentation:** Gordon Conference on Hormonal Carcinogenesis, Tilton, New Hampshire.

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